A STUDY OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS AND EXTENDED SPECTRUM BETALACTAMASE PRODUCING BACTERIA IN DIABETIC FOOT ULCERS IN A TERTIARY CARE HOSPITAL

Dissertation Submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

in partial fulfillment of the regulations
for the award of the degree of

M.D. (MICROBIOLOGY)
BRANCH – IV

GOVT. KILPAUK MEDICAL COLLEGE & HOSPITAL
THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI, INDIA.

SEPTEMBER 2010

DECLARATION

I solemnly declare that this dissertation "**A STUDY OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS AND EXTENDED SPECTRUM BETALACTAMASE PRODUCING BACTERIA IN DIABETIC FOOT ULCER IN A TERTIARY CARE HOSPITAL"** is the bonafide work done by me at the Department of Microbiology, Govt. Kilpauk Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. THYAGARAJAN RAVINDER M.D**, Professor of Microbiology and **Dr. RADHIKA KATRAGADDA M.D.,** Associate Professor of Microbiology ,Govt. Kilpauk Medical College, Chennai-600 010.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in September 2010.

Place : Chennai.
Date **Dr.Uma S. Pandian**

CERTIFICATE

This is to certify that this dissertation entitled **"A STUDY OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS AND EXTENDED SPECTRUM BETALACTAMASE PRODUCING BACTERIA IN DIABETIC FOOT ULCER IN A TERTIARY CARE HOSPITAL"** is the bonafide original work done by **Dr. UMA S. PANDIAN,** Post graduate in Microbiology, under my overall supervision and guidance in the department of Microbiology, Kilpauk Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

Dr. V. Kanagasabai, M.D.,
Dean
Kilpauk Medical College
Chennai-600010

Dr. Thyagarajan Ravinder M.D,
Prof. & H.O.D.
Department of Microbiology
Kilpauk Medical College
Chennai-600 010.

ACKNOWLEDGEMENT

My sincere thanks to **Dr. V. KANAGASABAI, M.D., DEAN.,** Government Kilpauk Medical College and Hospital, for giving me permission to commence this dissertation and use the resources of this Institution.

I am indebted and grateful **to Dr. THYAGARAJAN RAVINDER M.D,** Professor and Head of Department, Department of Microbiology,for his relentless efforts, valuable advice, excellent guidance and encouragement given to me throughout this study.

I owe my gratitude to **Dr. RADHIKA KATRAGADDA MD,** Associate Professor Department of Microbiology for her unflinching interest and motivation extended to me during my study.

I extend my gratitude to **Dr. K.V.LEELA M.D, D.G.O,** Associate Professor Department of Microbiology for her timely advice and encouragement.

I am grateful to **Prof. K. JEYANTHI. M.D.,** former Professor and HOD, Microbiology for her perpetual support, valuable advice and encouragement extended to me.

My sincere and special thanks to **Prof. NIYANTHRINI SRIDHAR, M.D.,** former HOD, Professor of Microbiology for her constant support, encouragement and timely help in carrying out this study.

I am grateful to **Prof. Dr. S. ANAND MOSES MD, HOD**, Institute of Diabetology Kilpauk Medical College and Hospital, for his valuable guidance given for my study.

I express my sincere thanks to **Prof. Dr. C. VIJAYLAKSHMI MS, Mch, former HOD**, Dept of Plastic Surgery Kilpauk Medical College and Hospital for her invaluable guidance for my study.

My sincere thanks to **Dr. S. MEENAKSHI, M.D.,** Assistant Professor of Microbiology, for keeping up my morale and for the enduring support and guidance provided when most needed.

I extend my sincere thanks to Assistant Professors **Dr. J. RAJA, M.D** and **Dr. SHANTHI MD**, Department of Microbiology for their help, support, interest and valuable hints.

My sincere thanks to **Mrs. RAMA VYDIANATHAN M..Sc., M.phil HOD,** Department of Industrial Biotechnology, and **Mr. MAGESH M.Sc** Research Scholor, Dr MGR Research and Educational Institute, Dr MGR Medical University, Maduravoyal, Chennai for their help in carrying out the molecular part of my work

I also thank all my department colleagues for their timely help, co-operation and support.

I express many thanks to all the technical staff and other staff members of the Department of Microbiology for their kind co-operation to carry out this work successfully.

I also extend my thanks to all the patients who participated in my study.

CONTENTS

INTRODUCTION

Diabetes Mellitus currently affects more then 19.4 crore people worldwide and the figure is expected to reach 33.3 crore by 2025[33]. India itself harbours over 3.5 crore diabetics which is the highest among the countries of the world and it is expected to touch 7.35 crore by 2025[33]. These staggering figures unfortunately have earned India the title of "DIABETIC CAPITAL OF THE WORLD".

Diabetic foot ulcers are estimated to affect about 25% of all diabetics during their lifetime accounting for 20% of diabetic related hospitalisation. The risk of lower extremity amputation is 15-46 times higher in a diabetic and diabetic foot ulcers precede 85% of all foot amputations . Nine out of ten amputations are instigated by an infection. It is the spark that leads to amputation though circulation is critically important, it only determines the level of amputation.[33]

The burden of diabetic foot ulcer is set to rise further in the future since contributory factors such as peripheral neuropathy and peripheral vascular disease are present in more than10% of diabetics at the time of diagnosis.[33] There is little awareness for foot care in patients and there is significant delay in seeking the treatment. Further a significant population in our country is rural and work in the fields barefoot, thus increasing the chances of further infection.

Prolonged or indiscriminate antibiotic use leads to drug resistance. Hence now we not only have multidrug resistant bacteria emerging but also fewer drugs which can be safely prescribed. With the indiscriminate usage of antibiotics there is today emergence of mutidrug resistant organisms, leading in this regard are Methicillin resistant Staphylococci, (MRSA) Beta lactamase producing gram negative bacilli (ESBL), vancomycin resistant Enterococci (VRE) etc.

Many of the MRSA strains are multidrug resistant and are susceptible only to glycopeptides ,antibiotics such as vancomycin. Therefore the knowledge of the prevelence of MRSA and their current antimicrobial profile becomes necessary in the selection of appropriate empirical treatment of these infections.

The third generation cephalosporin like Cefotaxime, which became widely used for the treatment of serious infection due to gram negative bacilli, in 1980s were also called as Expanded Spectrum Antibiotics. Due to extensive use of these antibiotics, bacteria soon developed resistance, by means of a new β - lactamases and because of their increased spectrum of activity against most of the β-lactam antibiotics including third generation cephalosporins, these enzymes were called Extended Spectrum Beta Lactamases (ESBL).

There is paucity of data on the frequency of the emerging resistant strains causing infection and the outcome of such infections among diabetic foot ulcers in India.

The present study aims to determine the microbiological and antimicrobial susceptibility profile of multidrug resistance organisms among diabetics.

AIMS AND OBJECTIVES

1. To isolate and identify bacterial pathogen from diabetic foot ulcer.
2. To study the antibiotic sensitivity pattern of the isolated bacterial pathogens.
3. To assess the risk factors predisposing to diabetic foot ulcers.
4. To characterise Methicillin resistant Staphylococcus aureus in the diabetic foot ulcer.
5. To characterise Extended spectrum betalactamase producers among the Gram negative pathogens.

REVIEW OF LITERATURE

India is home of nearly 33 million diabetics which is highest in the world out of which nearly 15% suffer from the dreaded sequel of diabetic foot[33]. Use of improper footwear and lack of knowledge regarding foot care attributes towards increase in the prevalence of diabetic foot.[33]

In patients with diabetes the probability of infection in foot ulcer is five times greater than non diabetic patients.[34] The reduced availability of essential blood components in diabetic wound, severely impair healing. When deprived of oxygen cells involved in wound healing die leading to the accumulation of devitalized tissue that provides an ideal environment for the growth of a diversity of micro organism. Additionally ischemia and a dry environment interfere with delivery and activity of polymorphonuclear cells in wound tissue, compromising the clearance of wound microflora [34].

PATHOPHYSIOLOGY

A variety of physiologic and metabolic disturbances conspire to place diabetic patients at high risk for foot wounds. The various predisposing factors include metabolic derangements, faulty wound healing, neuropathy and vasculopathy . Microbial colonization of wounds is inevitable usually with endogenous bacteria, but these are potentially pathogenic in the wound environment. The risk of wound infection increases when local condition favour bacterial growth rather than host defence. Avoiding infection in a wound is most effectively achieved by clearing devitalized tissue and foreign bodies and ensuring adequate tissue perfusion.

Immunologic disturbances are also an important predisposing factor for infection. Among the defects in host immune defences associated with diabetes are impairments of polymorphonuclear leukocyte functions, intracellular killing, and chemotaxis. Many of these immunodceficencies are directly related to the metabolic perturbations caused by poorly controlled diabetes. The prevalence of these defects appears to be correlated, at least in part with the adequacy of glycaemia control. Ketosis in particular impairs leukocyte function. Evidence suggests that in diabetic patients cellular immune responses and monocyte function are reduced.[33]

Poor granuloma formation prolonged persistence of abscesses and impaired wound healing are further accompaniments of diabetes that may predispose to infectious complications. Diabetic patients also appear to have a higher rate of carriage of Staphylococcus aureus in their anterior nares and subsequently on the skin.[9] This colonization may predispose to skin infections with this virulent pathogen when there is a break in the protective dermal surface. In addition several types of skin disorders as well as skin and nail fungal (Tinea) infections, disproportionately plague diabetic patients. In one study evidence of pedal fungal infection was found in over 80% of people with long term type I diabetes. Fungal infections provide breaks in the cutaneous envelope that then offer potential sites for bacterial invasions. The unique anatomy of the foot is among the reasons that infection is potentially so serious in this location. The structure of the various compartments, tendon sheaths and neurovascular bundles tends to favour the proximal spread of infection[36].

Poor blood glucose control contributes to increased mortality and mobility since hypoglycaemia is contributory to the development of infection and increased susceptibility to tissue injury[31]. Neuropathy on the other hand predispose a diabetic to unrecognizable injury due to loss of sensation It is therefore important to educate patients on preventive foot care and remind physicians to examine the foot carefully on every visit.[31]

MICROBIOLOGY OF FOOT ULCERS

Many studies have reported on the bacteriology of diabetic foot infections (DFIs) over the past 25 years but the results have varied and have been contradictory. A number of studies have found that Staphylococcus aureus is the main causative pathogen but two recent investigations reported a predominance of gram negative aerobes.[32] The role of anaerobes is particularity unclear because in many studies specimens are not collected or cultured properly to recover these organism[32].

Previous studies have shown that when optimal specimen collection, transport and culture technique are used, multiple organism are usually recovered from DFIs. Furthermore some studies suggest that the interaction of organism within these polymicrobial mixture lead to the production of virulence factors such as hemolysins proteases and collegenases as well as short chain fatty acids, that cause inflammation, impede wound healing and contribute to the chronicity of the infection in such mixtures, biofilm that

impede the penetration of antimicrobial agents into the infected site may also form, thus the presence of multiple species can have important clinical implication that should not be over looked [69]

The skin being coated with bacteria present in a harmless associations will rapidly colonize disrupted epithelium and microbial multiplication ensues, with local tissue destruction and release of bacterial toxins inciting a host response the wound is now defined as **infected.**

Infection may either follow colonization or occur as a primary event for example in the setting of acute trauma. Infection involves the invasion of host tissues by microorganisms (Pathogens) with a subsequent host inflammatory response (erythema, induration, pain or tenderness ,warmth loss of function and purulent secretions.)In diabetes the colonizing flora become more complex; virulent aerobic gram-positive cocci notably S. aureus and B-hemolytic streptococci may flourish. Antibiotic therapy can also alter the colonizing flora of skin or wounds, favouring organisms that are resistant to the agent administered.

Lesions that have been infected for a short time tend to be monomicrobial and to be caused by gram positive pathogens. Chronic wounds develop complicated flora with aerobic gram negative rods, anaerobes (gram positive and negative) and enterococci in addition to the gram positive aerobes. Fungi (including Candida and Tinea species) also appear to disproportionately colonize the skin of diabetic patients.[69]

Foot infections are generally polymicrobial. Cultures from these infections yield on average 4.1 - 5.8 bacterial species per culture. Both gram positive cocci and gram negative rods are commonly isolated from a single lesion, and in 40% of infections both aerobic and anaerobic organisms are recovered.[69] Individual cultures have yielded on average 2.9 - 3.5 aerobes and 1.2 – 2.6 anaerobes S. aureus, streptococci and facultative gram negative bacilli Proteus species, Enterobacter species, Escherichia coli and Klebsiella species) are the predominant aerobic pathogens in these infections.[71] Among the anaerobes Peptostreptococcus species, Prevorella species and Bacteroides species including those of the B.fragilis group are recovered frequently. Clostridium species are recovered infrequently.

The spectrum of bacterial species recovered from foot infections, especially those that are limb threatening, can be dramatically altered by prior failed antimicrobial therapy whereas Pseudomonas aeruginosa, Acinetobacter species and other antibiotic resistant facultative gram-negative bacilli are uncommon in previously untreated infections these organisms are not infrequent isolate from infected chronic ulcers . Similarly methicillin-resistant S.aureus may be encountered commonly in patients with chronically infected foot ulcers that have persisted in spite or multiple prior courses of antimicrobial therapy. These resistant bacteria are probably acquired nosocomially or alternatively emerge from endogenous flora during the repetitive hospitalization and antibiotic treatment of patients with non healing foot ulcers.

Accordingly, when selecting an antimicrobial regimen to treat a foot infection in a patient who has had multiple hospitalizations and prior courses of antibiotics, physicians should anticipate the presence of antibiotic resistant pathogens.(69)

The role of relatively avirulent bacteria many of which are part of skin flora that are often isolated from cultures of specimens obtained through an ulcer is uncertain. Staphylococcus epidermidis has been recovered usually in conjunction with other bacteria from 15-35% of these infections and may not reflect ulcer colonization.(69) On the other hand S.epidermidis has been isolated from reliable foot specimens with similar frequency to S.aureus suggesting that these organisms may be pathogens in some patients .Enterococci variants streptococci and Corynebacterium species, organisms that are often considered contaminants and not pathogens when isolated from skin and soft tissue infections, are among the isolates recovered frequently from polymicrobial limb threatening foot infections. When recovered from specimen in conjunction with typical pathogens these organisms are often disregarded as contaminants. Often, foot infections respond to therapy with antimicrobials that are active in vitro against the pathogens but not against these presumed contaminants. Although these observations support the designation of these organisms as contaminants, they could also indicate that with the eradication of major pathogens, host defences and surgical debridement can control these less virulent organisms.(69)

THE INFECTED FOOT ULCER

Diabetic foot ulcer are not spontaneous ulcer but result from the interplay of various factors like peripheral neuropathy, autonomic neuropathy and peripheral vascular disease are superimposed with alteration in the plantar pressure, defective footwear and limited joint mobility. Cell mediated immunity is most affected with abnormality of polymorphonuclear leucocytes, monocytes and lymphocytes.[33]

Clinical assesment of the ulcer include description of location, appearance, extent, depth temperature and odour. Infected wounds may appear purple or red or even brown or black, depending on the pathogen and its aetiology and their drainage may be serous, hemorrhagic or purulent. Induration of the skin and swelling denotes infection which usually raises skin temperature. Foul odour of the ulcer may denote infection by a specific pathogen such as Proteus spp, Pseudomonads or anaerobes, a mixed infection or fungi or simply a necrotic process. Wound infection are categorized as mild, moderate or severe.

Mild infections are superficial infections confined to the skin and the subcutaneous fat with minimal or no purulence or cellulites, moderate infections are deep and may involve fascial muscle, tendon, joints or bones. They may present as cellulitesA of 0-2 cm in diameter or as a plantar abscess and they may cause systemic symptoms. They impose a certain risk of

amputation. Severe infection of a foot ulcer is a deep infection with more than 2cm of cellulites lymphangitis, gangrene and causing systemic toxicity [36]. All diabetic foot are classified and grouped according to **Wagner grading system**. In the Wagner's classification system foot lesions are divided into six grades based on the depth of the wound and the extent of tissue necrosis. Failure of the Wagner's classification to specially address infection and ischemic within each grade has been recognized and a hybrid scheme has been developed to account for their important attributes to foot ulcers.

A simplified system which only attached modifies for ischemia (A) and infection (B) is currently followed universally.

MODIFIED WAGNER's CLASSIFICATION SYSTEM [35]

GRADE LESION

O - No open lesion may have deformity or cellulites
0 - A ischemic
0 - B infected
1 - superficial ulcer
1 - A ischemic
1 - B infected
2 - deep ulcers to tendon or joint capsule
2 - A ischemic
2 - B infected
3 - Deep ulcers with abscess and osteomyelitis
3 - A Ischemic
3 - B infected
4 - Localized gangrene- forefoot or heel
4 - A Ischemic
4 - B infected
5 - Gangrene or entire foot
5 - A Ischemic
5 - B Infected.

The decision regarding proper management of diabetic foot infection is a difficult one and still a matter of debate. While optimal therapy is yet to be established most authors agree that the management of these infection require isolation and identification of the microbial flora, appropriate antibiotic therapy according to the susceptibility pattern, proper selection and identification of the chronic complications and proper surgical intervention with adequate glycaemia control. As most of the diabetic foot infection are polymicrobial in nature, mixed organisms are frequently encountered with emergence of resistance among organisms against the commonly used antibiotics due to their indiscriminate use.

Methicillin resistant Staphylococcus aureus(MRSA) has been a pathogen of concern in patients with Diabetic foot infections(DFIS) for almost two decades. In fact the first two isolates of vancomycin resistant MRSA strains were from diabetic patients with foot lesions, more recently the emergence of community acquired MRSA has been noticed [32]. Recent studies from India have reported prevalence of Extended spectrum betalactamases(ESBL) producers to be as high as 68%, Babypadmini et al (2004) have shown 40% of K pnemonia isolates and 41% of E coli isolates to be ESBL producers, the studies from Brazil show only 6% of ESBL producers in diabetic foot infections. Antipapal et al have however reported 54.5% isolates to be ESBL producers which have caused diabetic foot ulcers. Thus the prevalence of MRSA AND ESBL constitutes a serious threat to the current antibiotics therapy leading to treatment failure and consequent escalation of costs. [39]

THE EMERGENCE OF MRSA

Resistance to methicillin and other penicillin was first observed in S. aureus soon after methicillin was introduced into clinical use in Britain. The methicillin resistant strains isolated in Britain at that time came from hospitalized patients and were multiple antibiotic resistant. They belonged to phage group III and their resistance to methicillin was heterogenous affecting only a minority of cell populations.

After the mid 1970s large out breaks of infection by MRSA were recorded in many hospitals. Many of these out breaks appear to have been caused by a single epidemic strain that was transferred between hospitals by the movement of patients. A nomenclature has been developed to differentiate strains associated with epidemic MRSA from those associated with sporadic infection .

MRSA in INDIA

One of the earliest report from India was in 1982 when an incidence of 6.6% was reported by Bhatia et al. In 1996 pullimood et al from Vellore reported an MRSA isolation rate of 24%, Uday shankar et al reported the figure as 20% from Pondicherry.

A systematic study done in Delhi over six month period by Krishna prakash et al in 2001 reported on MRSA isolation rate of 38.6%, in 2003 hanumanthappa et al reported an isolation rate of 43% a higher rate of 54.85% has been reported in the same year by Anupurba et al from Banaras.

Patients with MRSA have also been shown to be at increased risk for delayed treatment. Inappropriate antibiotic choices also has been associated with higher hospital mortality rate[4] furthermore MRSA strain cause therapeutic problems because of their ability to develop resistance to other classes of antibiotics including glycopeptides.[16]

MECHANISM OF RESISTANCE

Melhicillin resistance requires the presence of the chromosomally localized mec A gene. The mec A gene is responsible for the synthesis of penicillin binding protein 2a (PBP 2a; also called PBP2) – a 78 KDa protein penicillin binding protiens (PBPS) are membrane bound enzyme that catalyze the transpeptidation reaction that is necessary for cross linkage of peptidoglycan chains. PBP 2a substitute for the other PBP s and because of its low affinity for all beta lactam antibiotics, it enables Staphylococcus to survive exposure to high concentration of these agents. Thus resistance to methicillin confers resistance to all betalactam antibiotics including cephalosporins. PBP 2a differs from other PBPs in that the active site blocks binding of all Beta-lactams but allows the transpeptidation reaction to proceed.

The mecA is part of a genomic island designated staphylococcal cassette chromosome (SCCmec) To date four different SCCmec elements have been characterized. Health care associated MRSA(HA- MRSA) strain tend to carry SCC mec type I, II and III while the type IV element is generally carried by community associated MRSA (CA MRSA)[21]

Evidence available suggest a high frequency of a single clonal subgroup ST 239 to be the isolated strain from nine Asian countries from Saudi Arabia to the Philippine [19] This genotype has been characterized using Multilocus sequence typing (MLST) as HA- MRSA isolated from mainland Asia. Two recent reports noted an association between ST 239 and increased virulence.[14]

Community associated MRSA (CA MRSA) has been defined both epidemiologically and molecularly. They have been typed as USA 300 and 400 using pulsed field gel electrophoresis CA MRSA typically also has a characteristic antibiotic susceptibility pattern, carries specific virulence factor such as the panton - valentine leukocidin (PVL) and posesses a specific SCC mec type IV.[8,17]

LABORATORY DIAGNOSIS OF MRSA:

Detection of S.aureus

Isolation of staphylococci from clinical specimen performed using blood agar plates followed by incubating at 35c - 37 C for 18 - 24 hrs . Most staphylococcal species will produce abundant growth and the colonies are usually 1-3 mm in diameter, golden yellow pigmented, hemolytic, circular, smooth and raised with a butyrous in consistency.

S.aureus is identified by its colony morphology. Grams stain demonstrates the presence of gram positive cocci in clusters and it also gives a positive catalase test.

S.aureus strains are usually identified using phenotypic and genotypic methods:

Conventional methods of detection of MRSA:

1. DISC DIFFUSION METHODS

Oxacillin disc (lug)[50]

Disc diffusion test is performed with lug of oxacillin per disc on 25 ml of Mueller-Hinton agar with NaCl supplementation. The zone of inhibition is determined after 24hrs of incubation at 37.c The zone size is interpreted according to CLSI guidelines.

SUSCEPTIBLE - > 13 mm

INTERMEDIATE - 11-12mm

RESISTANT - <10mm

Cefoxitin disc(30ug) diffusion test[1]

Cefoxitin, a cephamycin is a more potent inducer of the mec A regulatory system than are the penicillins. Several groups of investigators have reported that the results of cefoxitin disc diffusion tests correlate better with the presence of mec A than do the results of disk diffusion tests using oxacillin.[50]

Based on a large number of data CLSI subcommittee on ANTIMICROBIAL SUSCEPTBILITY TESTING (CLSI – AST) adopted the use of the cefoxitin disc diffution test for predicting mec A mediated oxacillin resistance in Staphylococci and added a section for the test to table 2 C(the

Staphylococcal table) in both the disc diffusion (M2) and MIC (M7) SECTION of CLSI documents M100 – S14[1]

The test is performed with 30 ug of cefoxitin per disc on 25ml Muller Hinton agar without NaCl supplementation. The zone of inhibition is determined after 24 hrs of incubation at 37C.The zone size is interpreted according to CLSI guidelines.

Susceptible	>19mm
Resistant	<20mm

2) MIC DETERMINATION

1) Agar dilution method [24]

The inoculum is prepared by emulsifying portions of 4-5 discrete colonies into 4-5 ml of nutrient broth turbidity adjusted by Mc Farlands standard 0.5. An aliquot of 1 in 20 dilution with normal saline (0.0001 ml) is used as the final inoculum. The concentrations of oxacillin used is 32ug-0.015 ug/ml. The plates are dried before they are inoculated. The inoculum is applied as a spot of about 5-8 mm in diameter. A platinum loop calibrated to deliver 0.001 ml of inoculum is used. The plates are then incubated for 24hrs at 37.c. The endpoint is the concentration of the antibiotic completely inhibiting the growth. The results are as the MIC value in ug/ml

Susceptible	< 2ug/ml
Resistant	> 4ug/ml

2) broth dilution method

Micro dilution method

Macro dilution method

Serial dilution of oxacillin is added to Mueller Hinton broth with 4% NaCl. A young peptone water culture of S aureus corresponding to 0.5 Mc farland turbidity is used as the inoculum and it is incubated at 33-35 C for 24 hrs.

Oxacillin MIC < 2 ug/ml -Susceptible

> 2ug/ml – resistant

3) E – Test method

E – Test also known as the epsilometer test is an exponential gradients testing methodology where E in E test refers to the greek symbol epsilon. The E test which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium. A pre defined stable antimicrobial gradient is present on a thin inert carrier strip. When this E test strip is applied onto an inoculated agar plate there is an immediate release of the drug. Following incubation a symmetrical inhibition eclipse is produced The intersection of the inhibitary zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range with inherent precision and accuracy.

4) Latex agglutination test for detection of PBP 2a

A loop full of bacterial cells are suspended in 200 ul of extraction reagent and subsequently lysed by boiling for 3 min, after cooling to room temperature 50ul of another extraction reagent is added to 200ul of the lysate and mixed well. After 1 min of centrifugation at 1500 x g rpm 40 ul of the supernatant is used for testing agglutination with sensitised latex particles (1

drop) The test card is rotated for 3 min and the resulting agglutination patterns are usually read.

NEWER METHODS

SCREENING TEST

Mannitol salt agar- cefoxitin screening medium[2]

Mannitol salt agar(MSA) plates are prepared with cefoxitin at concentrations of 2,3 or 4 mg/liter. The test strains are applied by using fresh overnight cultures on blood agar matched to 0.5 Mc farland standard. Incubation is carried out at 35 c to 37c for 48 hrs and results are read at 18 hrs and 48 hrs.

The growth of any visible colonies after incubation is recorded as positive result.

CHROMagar MRSA[9]

It combines primary isolaton from clinical specimen with direct identification of MRSA in one step.On this agar MRSA isolates appear as light mauve to mauve colonies at 16 to 18 hrs of incubation. Others will appear as white or beige or grow poorly.

GENOTYPIC METHODS:

1) Multiplex PCR for MRSA

a) Detection of mecA and fem B genes
b) Detection of mecA and Coag genes
c) Detection of mecA and ccr genes
d) Detection of mecA and nuc genes
e) Detection of toxin genes (PvL genes)

2) REAL – TIME PCR

Real time PCR assays are used to detect MRSA directly from positive blood culture bottles .The assay differentiates MRSA into clusters on the basis of melting curve analysis.

3) Pulsed- fieid gel eletrophoresis:[5]

A bacterial pellet obtained from an overnight grown culture of a single colony is processed and the restriction fragments are seperatedon gel. Gel is stained with ethidium bromide and photographed under UV light.

Strain relatedness among CA-MRSA and HA-MRSA isolates can be investigated.

4) Multi Locus Sequence Typing (MLST)[5]

MLST is useful for investigating the clonal evolution of MRSA It is based on sequence analysis from seven S.aureus housekeeping genes i.e.,arcC,aroE,glpF,gmk,pt,tpi and yqiL.Each isolate is defined by all the alleles of the seven genes. This results in an allelic profile or gene sequence type(ST)

5) Microarray Analysis:[10]

Multiplex PCR products can be used as hybridization samples. After hybridization at the test site of the microarray, fluorescence detection is performed automatically by the instrument images of the array is then captured automatically and analysed using the image analysis software of the instrument.

MANAGEMENT OF MRSA INFECTIONS

Vancomycin is the drug of choice for serious infection caused by methicillin resistant Staphyloccocus aureus.[3]

Vancomycin and teicoplanin are glycopeptide antibiotics used to treat MRSA infections. Teicoplanin is a structural congener of vancomycin that has a similar activity spectrum but a longer half-life. Because the oral absorption of vancomycin and teicoplanin is very low, these agents must be administered intravenously to control systemic infections. Treatment of MRSA infection with vancomycin can be complicated, due to its inconvenient route of administration.

Several newly discovered strains of MRSA show antibiotic resistance even to vancomycin and teicoplanin. These new evolutions of the MRSA bacterium are called Vancomycin intermediate-resistant *Staphylococcus aureus* (VISA). Linezolid, quinupristin/dalfopristin, daptomycin, and tigecycline are used to treat more severe infections that do not respond to glycopeptides such as vancomycin.

Treatments in clinical trials

It has been reported that maggot therapy to clean out necrotic tissue of MRSA infection has been successful. Studies in diabetic patients reported

significantly shorter treatment times. Many antibiotics against MRSA are in phase II and phase III clinical trials. eg:

- Phase III : ceftobiprole, Ceftaroline, Dalbavancin, Telavancin, Aurograb, torezolid, iclaprim...
- Phase II : nemonoxacin.

Pre-clinical research

An entirely different and promising approach is phage therapy which in mice had a reported efficacy against up to 95% of tested *Staphylococcus* isolates. On May 18, 2006, a report in *Nature* identified a new antibiotic, called platensimycin, that had demonstrated successful use against MRSA.

Prevention and infection-control strategies

Screening programs

Patient screening upon hospital admission, with nasal cultures, prevents the cohabitation of MRSA carriers with non-carriers, and exposure to infected surfaces.

Surface sanitizing

Alcohol has been proven to be an effective surface sanitizer against MRSA.

Quaternary ammonium compounds can be used in conjunction with alcohol to extend the longevity of the sanitizing action. The prevention of nosocomial infections involves routine and terminal cleaning.

Hand washing

Although alcohol-based rubs remain somewhat effective, a more effective strategy is to wash hands with running water and an anti-microbial cleanser with persistent killing action, such as Chlorhexidine.

Decolonization

After the drainage of boils or other treatment for MRSA, patients can shower at home using chlorhexidine or hexachlorophene antiseptic soap from head to toe, and apply mupirocin (Bactroban) 2% ointment inside each nostril twice daily for 7 days, using a cotton-tipped swab. Household members are recommended to follow the same decolonization protocol.

Proper disposal of hospital gowns

Used paper hospital gowns are associated with MRSA hospital infections, which could be avoided by proper disposal.

Restricting antibiotic use

Glycopeptides, cephalosporins and in particular quinolones are associated with an increased risk of colonisation of MRSA. Reducing use of

antibiotic classes which promote MRSA colonisation, especially fluoroquinolones is recommended in current guidelines.

The Emergence of ESBL

Microbial resistance through extended-spectrum B-lactamase (ESBL) was first reported in the early 1980s in Europe and subsequently in the United States soon after the introduction of third-generation cephalosporins in clinical practice [58]. Today, this resistance mechanism has emerged globally and ESBL-producing Enterobacteriaceae are recognized world wide as nosocomial pathogens of major importance [19, 28] Many clinical microbiology laboratories have problems with the detection of ESBL-mediated resistance, and the recent emergence and spread of novel types of community-acquired ESBLs, such as the CTX-M enzymes have created additional challenges that further complicate the detection of this resistance mechanism.[58]

Resistance to third generation oxyimino-cephalosporins is mediated by extended spectrum B-lactamase enzymes (ESBLS) which are derivatives of narrow spectrum TEM and SHV B-lactamases capable of hydrolyzing oxyimino-cephalosporins (but not cephamycin such as cefoxitin and cefotetan). They are inhibited by clavulanic acid and are placed into Bush's functional group 2be. The diversity for ESBLs results in various susceptibility profiles with different B-lactam antibiotics. Some variants (TEM-3 and-4) give high-level resistance to all second and third generation cephalosporin's while other

variants (TEM-10-12, and-26) give obvious resistance to ceftazidime but give moderate resistance to cefotaxime, ceftriaxone, and to the fourth generation cephalosporins. ESBL production is known commonly to occur in E. coli and Klebsiella but have also been found in other members of the Enterobacteriaceae family. [68]

These enzymes are the result of mutations of Temorina (TEM-1 and TEM-2) and Suph hydryl variable (SHV-1) enzymes, usually plasmid mediated and are commonly found in the Enterobacteriaceae family. Some derivatives of TEM and SHV, which are not inhibited by clavulanic acid, are known as inhibitor resistant TEM (IRT) and the AmpC Class of enzymes, which are intrinsically resistant to clavulanic acid and are causing great concern as Carbapenems are the only antibiotic effective against such strains.[67]

Bacterial resistance to carbapenems mediated by acquired carbapenemases represents an important problem worldwide [10,15]. These carbapenemases can be classified into three molecular classes according to the Ambler scheme (15)A (Penicillinases), B (metallo-B-lactamases (MBLs)and D (Oxacillinases). The Mettalobetalactamases(MBL) producing variants of the types IMP, VIM, SPM-1, AND GIM-1 are thus far some of the most clinically relevant due to their ability to confer broad-spectrum B-lactam resistance, the unavailability of clinically useful inhibitors and their potential for rapid and generalized dissemination [23]. This worrying situations prompts an early recognition of MBL producers for infections control and prevention of their –

generalized spread, in particular among emerging gram-negative, glucose non fermentative pathogens such as Pseudomonas spp. and Acinetobacter baumamii [15, 21]

In recent years CTX-M extended-spectrum ß-lactamases (ESBLs) have very rapidly disseminated and are now frequently reported from countries all over Europe and much of Asia. More than 50 $bla_{\text{CTX-M}}$ genotypes are described.

Epidemiological reports demonstrate that some enzymes are more frequently reported than others, that predominant enzyme type varies with country and that diverse CTX-M types often exist within a single country.

In **India**, the very first report of the presence of CTX-M-producing Enterobacteriaceae came from New Delhi. Six isolates from 2000 were investigated, all of which were found to be unrelated and all produced CTX-M-15. Since then several Indian surveys have reported the presence of ESBLs in clinical isolates based on phenotypic tests. Recent reported rates vary widely (12.6–71%), with most studies reporting a prevalence rate of around 50% and upwards. Considering a population of ~1.1 billion in India, this represents a very large reservoir of resistance genes [61].

CLASSIFICATION OF BETA- LACTAMASE

Classification schemes for bacterial beta-lactamases

Bush-Jacoby-Medeiros group	1989 Bush group	Richmond-Sykes class	Mitsuhashi-Inoue type	Molecular class	Preferred substrates	Inhibited by:CA	EDTA	Representative enzymes
1	1	Ia, Ib, Id	CSase	C	Cephalosporins	-	-	AmpC enzymes from gram-negative bacteria; MIR-1
2a	2a	Not included	PCase V	A	Penicillins	+	-	Penicillinases from gram-positive bacteria
2b	2b	III	PCase I	A	Penicillins, cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	2b'	Not included except K1 in class IV	CXase	A	Penicillins, narrow- & extended-spectrum cephalosporins, monobactams	+	-	TEM-3 to TEM-26, SHV-2 to SHV-6, Klebsiella oxytoca K1
2br	Not included	Not included	Not included	A	Penicillins	+/-	-	TEM-30 to TEM-36, TRC-1
2c	2c	II, V	PCase IV	A	Penicillins, carbenicillin	+	-	PSE-1, PSE-3, PSE-4
2d	2d	V	PCase II, PCase III	D	Penicillins, cloxacillin	+/-	-	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	2e	Ic	CXase	A	Cephalosporins	+	-	Inducible cephalosporinases from Proteus vulgaris
2f	Not included	Not included	Not included	A	Penicillins, cephalosporins, carbapenems	+	-	NMC-A from Enterobacter cloacae, Sme-1 from Serratia marcescens
3	3	Not included	Not included	B	Most b-lactams, including carbapenems	-	+	L1 from Xanthomonas maltophilia, CcrA from Bac-teroides fragilis
4	4	Not included	Not included	ND[c]	Penicillins	-	?	Penicillinase from Pseudomonas cepacia

a Csase, cephalosporinase; PCase, penicillinase; CXase, cefuroxime-hydrolyzing b-lactamase.

b CA, clavulanic acid.

c ND, not determined.

Detection of ESBLs:

I. Screening for ESBL producers-

(i) Disc diffusion method:

The CLSI has proposed disc diffusion methods for screening for ESBL production by Klebsiellae, *Escherichia coli*, and *Proteus mirabilis*. Laboratories using disc diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters, which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone is used. However, the use of more than one of these agents for screening improves the sensitivity of detection. If any of the zone diameters indicate suspicion for ESBL production, phenotypic confirmatory tests should be used to ascertain the diagnosis.

(ii) Double-disc diffusion test

In the late 1980s, French investigators described a disc diffusion test in which synergy between cefotaxime and clavulanate was detected by placing a disc of amoxicillin/clavulanate (20 μg/10 μg) and a disc of cefotaxime (30 μg), 30 mm apart (center to center) on an inoculated agar plate. A clear extension of the edge of the cefotaxime inhibition zone toward the disc containing clavulanate was interpreted as synergy, indicating the presence of an ESBL;

Clinical Laboratory Standards Institute (CLSI) Recommended Methods for ESBL Detection:

II. Phenotypic Confirmatory Tests for ESBL Production

(i) Cephalosporin/clavulanate combination discs.

The CLSI advocates use of cefotaxime (30 μg) or ceftazidime discs (30 μg) with or without clavulanate (10 μg) for phenotypic confirmation of the presence of ESBLs in klebsiellae and *Escherichia coli*. The disc tests are to be performed with confluent growth on Mueller- Hinton agar. A difference of 5 mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate disc is taken to be phenotypic confirmation of ESBL production.

(ii) Broth microdilution

Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 μg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 μg/ml), cefotaxime (0.25 to 64 μg /ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4 μg/ml). Again it should be emphasized that both ceftazidime and cefotaxime should be used. A 3-twofold-serial-dilution decrease in MIC of either cephalosporin in the presence of clavulanic acid is compared to its MIC when tested alone.

Implications of positive phenotypic confirmatory tests

According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins, cefoxitin, and cefotetan) and aztreonam, regardless of the MIC of that particular cephalosporin.

III. Commercially Available Methods for ESBL Detection

(i) Etest for ESBLs

AB Biodisk (Solna, Sweden) produces plastic drug-impregnated strips, one end of which contains a gradient of ceftazidime (MIC test range 0.5 to 32 μg/ml) and the other with a gradient of ceftazidime plus a constant concentration of clavulanate (4 μg /ml). The reported sensitivity of the method as a phenotypic confirmatory test for ESBLs is 87 to 100% and the specificity is 95 to 100%. The manufacturer currently recommends a 8-fold reduction in cephalosporin MICs in the presence of clavulanate.

(ii) Vitek ESBL cards

Vitek ESBL test (bioMerieux Vitek, Hazelton, Missouri) utilizes cefotaxime and ceftazidime, alone (at 0.5 μg/ml), and in combination with clavulanic acid (4 μg/ml). Inoculation of the cards is identical to that performed for regular Vitek cards. Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4 to 15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin alone, indicates a positive result. Sensitivity and specificity of the method exceed 90%.

(iii) MicroScan panels

Dade Behring MicroScan (Sacramento, Calif.) produces dehydrated panels for microdilution antibiotic susceptibility testing.

(iv) BD Phoenix Automated Microbiology System

The Phoenix ESBL test uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ESBLs. Results are usually available within 6 hours.

In a study[4] the test sensitivities for MicroScan ESBL plus ESBL confirmation panel was 100%, VITEK 1 GNS-120 99%, Etest ESBL 97% and BD BBL Sensi-Disk ESBL Confirmatory Test disks 96%.

(v) Molecular ESBL detection techniques

Test	Advantages	Disadvantages
DNA probes	Specific for gene family (e.g., TEM or SHV)	Labor intensive, cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of TEM or SHV
PCR	Easy to perform, specific for gene family (e.g.,TEM or SHV)	Cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of TEM or SHV
Oligotyping	Detects specific TEM variants	Requires specific oligonucleotide probes, labor intensive, cannot detect new variants
PCR-RFLP	Easy to perform, can detect specific nucleotide changes	Nucleotide changes must result in altered restriction site for detection
PCR-SSCP	Can distinguish between a number of SHV variants	Requires special electrophoresis conditions
Nucleotide sequencing	The gold standard, can detect all variants	Labor intensive, can be technically challenging, can be difficult to interpret manual methods
Real Time PCR	Rapid identification, minimal cross contamination.	Expensive, technical skill required

TREATMENT OF INFECTIONS WITH ESBL-PRODUCING ORGANISMS

ESBLs

Strains producing only ESBLs are susceptible to cephamycins and carbapenems in vitro and show little if any inoculum effect with these agents.

For organisms producing TEM and SHV type ESBLS, apparent in vitro sensitivity to cefepime and to piperacillin/tazobactam is common, but both drugs show an inoculum effect, with diminished susceptibility as the size of the inoculum is increased from 10^5 to 10^7 organisms.

Strains with some CTX-M type and OXA type ESBLs are resistant to cefepime on testing, despite the use of a standard inoculum.

Inhibitor-Resistant B-Lactamases

Although the inhibitor-resistant TEM variants are resistant to inhibition by clavulanic acid and sulbactam, they remain susceptible to inhibiton by tazobactam and subsequently the combination of piperacillin/tazobactam.

AmpC

AmpC –Producing strains are typically resistant to oxyimino-beta lactams and to cephamycins and are susceptible to carbapenems how ever dimnished porin expression can make such a strain carbapenem-resistant as well.

Carbapenemases

Strains with IMP- VIM- and OXA type carbapenemases usually remain susceptible to aztreonam.

MATERIALS AND METHODS

This is a descriptive study for a period of one and half year from July 2008 to Dec 2009 to analyse the bacterial isolates from diabetic foot ulcers of patients attending the Department of diabetology, Kilpauk Medical college& Hospital.

A total of 100 diabetic foot ulcer patients from both sexes in age group between 35 to 82 years were studied during this period

INCLUSION CRITERIA:-

All diabetic patients with foot ulcers attending the department.

EXCLUSION CRITERIA:-

1. All non-diabetic patients with foot ulcers.
2. All diabetic patients with foot ulcers but on antibiotic therapy.

METHODOLOGY:-

Methodology includes

1. Collection of samples
2. Identification of the organisms and antibiotic sensitivity pattern
3. Selection of resistant strains
4. Detection of MRSA
5. Identification of mecA gene
6. Detection of ESBL production
7. Identification of CTX-M gene

SPECIMEN COLLECTION :

Swab from diabetic foot ulcer :

Wound area was wiped with sterile saline. Two sterile Swabs were rolled along leading edge of the wound, one for direct smear study and the other for aerobic bacterial culture. A bit of sough and tissue necrotic material from the base of the ulcer was inoculated into Robertson cooked meat broth.

SPECIMEN TRANSPORT :

The swabs were transported to the laboratory without delay.

SPECIMEN PROCESSING

Direct Gram Stained smear:

One of the swabs was used for direct smear study using Gram stain.

Aerobic culture :

The other swab was used to plate the following culture plates.

- 5% sheep Blood agar plate
- MacConkey agar plate
- Nutrient agar plate

Anaerobic culture:

After 48 hrs of incubation the Robertson cooked meat broth was observed for signs of growth and then streaked onto anaerobic blood agar plates with hemin and Vit K supplement along with antibiotics.

Incubation

The plates were incubated at 37degree centigrade for 24 hours for aerobic culture. Anaerobic plates were placed inside the polycarbonate jar along with Gaspak. Pseudomonas was used as biological control and methylene blue was used as a chemical indicator. The jar was left undisturbed for 48hrs.

Identification of pathogens:

After incubation the agar plates were analysed and every different looking colony was subjected to further processing . Gram stain was done for similar looking colonies. Pathogens were identified by growth on the basic plates and a battery of biochemical reactions as per standard laboratory protocol.

Antibiotic sensitivity testing:

Routine disc diffusion susceptibility testing of the strains was performed by modified Kirby Bauer Method in Mueller-Hinton agar medium. To standardize the inoculam density for a susceptibility test, a Barium sulphate turbidity standard equivalent to a 0.5 McFarland standard was used.

Tip of 3-5 representative colonies were picked up and put in 4-5ml of nutrient broth and was incubated at 35˚ C until it matched in density with 0.5 McFarland's standard, which corresponds to 150 million organisms/ml.

After 15 minutes of adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into it. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess broth from the swab.

Dried surface of Mueller Hinton agar plate was inoculated by swabbing over the entire sterile agar surface. The predetermined battery of antimicrobial discs were dispensed on agar plates and pressed down to ensure complete contact with the agar surface. Discs were distributed evenly so that they were not closer than 24mm from centre to centre. Plates were inverted and incubated at 37^{o}C for 16-18 hrs.

After 16-18 hrs of incubation each plate was examined. The resulting zones of inhibition was uniformly circular and there was a semi-confluent lawn of growth. The diameter of the zones of complete inhibition was measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter using a ruler which was held on the back of the inverted Petri plate.

The sizes of the zones of inhibition were interpreted by referring to the CLSI standards and reported as susceptible, intermediate, or resistant to the agents that have been tested.

Controls used with each batch:-

1. Escherichia coli ATCC 25922
2. Pseudomonas aeruginosa ATCC 27853
3. Staphylococcus aureus ATCC 25923

Detection of MRSA strains

Screening for MRSA:

Oxacillin disc (1ug)

Disc diffusion test is performed with lug of oxacillin disc was placed on 25 ml of Mueller-Hinton agar plate . The zone of inhibition is determined after 24hrs of incubation at 37.c the zone size is interpreted according to CLSI guideline

SUSCEPTIBLE	-	>13 mm
INTERMEDIATE	-	11-12mm
RESISTANT	-	< 10mm

Cefoxitin disc(30ug) diffusion test

The test is performed with 30 ug of cefoxitin per disc placed on 25ml Muller Hinton agar plate without Nacl supplementation. The zone of inhibition is determined after 24 hrs of incubation at 37C. The zone size is interpreted according to CLSI guidelines.

Susceptible	>19mm
Resistant	< 20mm

Quality control used for MRSA detection:

ATCC S.aureus 43300 (positive control)

ATCC S.aureus 25923 (negative control)

PCR FOR DETECTION OF mecA gene[16,17]

Preparation of cell lysate:

0.1ml of a an overnight peptone water culture was centrifuged for 30 seconds at 16000 rpm. Cells were then resuspended in 50 ul of lysostaphin (100ug/ml of water) and incubate at 37 c After 10 mins 50ul of proteinaseK solution (100ug/ml) and 150ul of TRIS buffer was added and reincubated for 10 mins. It was placed in boiling waterbath for 10 mins. 2ul of the supernatent was used as the DNA sample for PCR

Primer Sequence:

MecA forward primer – 5' AAAATCGATGGTAAAGGTTGGC-3'

MecA reverse primer - 5' AGTTCTGCAGTACCGGATTTGC-3'

PCR:

PCR cocktail was prepared according to the table tabulated below. To this cocktail 2 ul of the DNA sample is added PCR was set according to the following conditions:

1. Initial Denaturation for 5 mins at 94º C.
2. FOLLOWED BY 30 CYCLES
3. Denaturation for 30 secs at 94º C.
4. Annealing for 30 secs at 55º C.
5. Extension for 2min at 72◦C
6. 10 min at 72º C for amplification.

Cocktail Procedure:

S.no	Reagents	Amount to be added for 50ul
1.	WATER	38.5
2.	BUFFER	3
3.	Dntp	2
4.	TAQ POLYMERASE	0.5
5.	Primer-F +Primer-R	2 + 2
6.	DNA	2
7.	Total	50

Gel Documentation:

After the reaction, 10 µl. of the amplified samples were run on a 1.5 % agarose gel and electrophoresis done at 50 V with 1 x Tris Acetate EDTA buffer. Amplicons were visualized using Ethidium bromide staining and scored using 100 bp DNA ladder as reference.

Gels were viewed in a U - V gel documentation Unit and Photographed.

A 533bp corresponds to mecA gene specific oligonucleotides.[16]

Detection of ESBL

Screening for ESBL production

All gram negative isolates that were resistant to at least two 3rd generation cephalosporin namely cetriaxone(30mcg) and ceftazidime (30mcg) were considered to be probable ESBL producers and processed further for confirmation. [70]

(CLSI recommends MIC ≥ 2µg/ml for cefotaxime, ceftazidime, astreonam, ceftriaxone (or) cefpodoxime as potential ESBL producers).

2 indicators of ESBLs are

i) 8 fold reduction in MIC when 3rd generation cephalosporins are used with clavulanic acid.

ii) > 5mm increase in diameter of inhibition zone when using disc diffusion method with 3rd generation cephalosporin and clavulanic acid combined disc.

1) Double-disc diffusion test

In this test a disk of amoxicillin/ clavulanate (20 µg/10 µg) and a disk of cefotaxime (30 µg), 30 mm apart (center to center) was placed on an inoculated agar plate and incubated for 16 to 18hrs. A clear extension of the edge of the cefotaxime inhibition zone toward the disk containing clavulanate was interpreted as synergy, indicating the presence of an ESBL;

2. Phenotypic Confirmation Test

Lawn culture of the organism was made and 3rd generation cephalosporin, Ceftazidime (30µg) disc was tested alone and along with their combination for 10mg of clavulanic acid. Organisms with 5mm increase in zone of inhibition for ceftazidime / clavulanic acid (30µg/10µg) are confirmed as ESBLs.

3) MIC determination : Done by agar dilution method.

Preparation of media: Muller Hinton agar was prepared in tubes and autoclaved. It is then allowed to cool in a 50^{o}C water bath.

Serial dilution of the 3 generation cephalosporins - cefotaxime was prepared in sterile distilled water to give a final concentration ranging from 2μg – 2048μg/ml of agar.

After adding the drug to the medium at 50^{o} C it was mixed well and poured into sterile petridishes. (The media was used immediately otherwise potency of drugs will be affected. We can inoculate upto 12 different organisms in a single plate).

A control plate containing the test medium without the antibiotic was prepared for each series of test.

Inoculum Preparation:

At least 3-5 well isolated colonies of the same morphological type were selected from an agar culture plate. Top of each colony was touched with a loop and the growth was transferred into a tube containing 4-5ml of broth. The broth culture was incubated at 37^{o}C until it reaches 0.5 Mc Farland's standard (usually 2-6 hrs). This result in growth corresponding to 150 million organisms/ml.

Minimum inhibitory concentration was the lowest concentration at which no visible growth occurs.

Quality Control used for ESBL detection :

Klebsiella pneumoniae ATCC 700603 [as positive control]

Escherichia coli ATCC 25922 as negative control.

4) MIC Reduction test:

Isolates were tested for various concentrations of cephalosporin combined with 2ug/ml of clavulanic acid from 0.5ug to 2048ug / ml of agar and the MIC determined.

An eight fold reduction in the MIC of 3rd generation cephalosporins in the presence of clavulanic acid indicates production of ESBL.

5) PCR procedure for CTX- M gene[61]

Colony PCR technique was done in 13 isolates of Klebseilla spp.to identify the commonly expressed gene for resistance namely CTX-M

A single colony of the test organism was picked up from a nutrient agar plate and inoculated in 100 uL of distilled water, heated at 100◦c for 5min followed by centrifugation at 9000 rpm for 3min and then place in ice. 5ul of the centrifuged culture was added to the PCR cocktail mixture as given below.

Primer Sequence:

CTX-M/F: 5'-CGCTTTGCGATGTGCAG-3'

CTX-M/R: 5'-ACCGCGATATCGTTGGT-3'

PCR:

1. Initial Denaturation for 5 mins at 94º C.
2. FOLLOWED BY 30 CYCLES
3. Denaturation for 30 secs at 94º C.
4. Annealing for 30 secs at 55º C.
5. Extension for 1min at 72◦C
6. 10 min at 72º C for amplification.

COCKTAIL PROCEDURE:

S.no	Reagents	Amount to be added for 50ul
1.	WATER	35.5
2.	BUFFER	3
3.	Dntp	2
4.	TAQ POLYMERASE	0.5
5.	Primer-F +Primer-R	2 + 2
6.	DNA CULTURE	5
7.	Total	50

Gel Documentation:

After the reaction, 25 µl. of the amplified samples were run on a 1.5 % agarose gel and electrophoresis done at 50 V with 1 x Tris Acetate EDTA

buffer. Amplicons were visualized using Ethidium bromide staining and scored using 100 bp DNA ladder as reference.

Gels were viewed in a U - V gel documentation Unit and Photographed.

A 551 bp product corresponds to blaCTX-m gene specific oligonucleotides.[61]

Detection of Mettalobetalactamases (MBL) producing Pseudomonas strains

Screening for MBL producers

All pseudomonas isolates which showed a zone of inhibition less than 13mm around imipenam were considered to be carbapenam resistant [56]

Combined disc synergy test [56]

Two Imipenam (10ug) discs with a distance of 20mm between them were placed on the Mueller Hinton agar plate inoculated with the test strain.10ul of the inhibitor (EDTA) solution was added to one of the discs in the concentration of 750ug. After 24hrs of incubation period at 35degree C the increase (>7mm) of the inhibition zone obtained with the combined disk than the zone obtained with the Imipenam disc alone was taken as a positive test.

All the results obtained were statistically analysed and tabulated.

RESULTS

Patients with diabetic foot ulcers attending the Institute of Diabetology at Kilpauk Medical College and Hospital, Chennai were studied for the presence of Methicillin resistant Staphylococcus aureus and Extended spectrum betalactamase producing isolates in the diabetic foot ulcers. The study was done between July 2008 and Dec 2009.

100 patients with diabetic foot ulcer were studied and 142 bacterial isolates from the ulcers were isolated, identified and analysed for their antibiotic sensitivity pattern. Resistant strains were identified and studied for Methicillin resistant Staphylococcus aureus (MRSA) and extended spectrum betalactamases (ESBL) producing bacteria by various methods.

The observations were recorded and analysed. The results are as follows:

CLINICAL DATA

Table - I

Demography of patients

N = 100

Characteristic	Number of Patients	Ratio
Male Female	53 47	1: 0.8

100 patients with diabetic foot ulcer were studied. Out of these 100 patients included in the study 53 were male and 47 were female. The male to female ratio was 1: 0.8

Table II

Age distribution

N = 100

Age	No	Male	Female
30-40	7	5	2
40-50	19	14	5
50-60	32	16	16
60-70	28	18	10
70-80	13	6	7
above 80	1	1	0

In our study the maximum number of patients were in their 5th and 6th decade. The mean age of these patients was 58.6 years and ranged from 36 to 81 years.

Table III

Family History Of Diabetes Mellitus

N = 100

Family History of diabetes mellitis	No.
PRESENT	70
ABSENT	30

Out of the 100 patients family history of diabetes was seen in 70 cases. The remaining 30 did not have diabetes in the family.

Table IV

Random Blood Glucose Levels

N = 84

above 200mg%	below 200mg%
27	57

Random Blood glucose levels were noted and 27 patients out of the 100 cases were found to have glucose levels above 200mg% and 57 cases were seen to be below 200mg%.The values for the remaining 16 cases were not available.

Table V

Risk Factors

N = 100

FACTORS	No.
Peripheral neuropathy	52
Hypertension	48
History of diabetic foot Ulcer	35
History of Amputations	25
History of Trauma	13
History of Retinopathy	6
Peripheral vascular disease	12

Peripheral neuropathy was found to be the most common(52%) comorbid condition among the 100 patients studied. History of amputation was seen in 25 cases.

Table VI

Wagner `S Classification of Diabetic Foot Ulcer

N = 100

Grade	No.
I	21
II	64
III	14
IV	1
V	0

MICROBIOLOGICAL DATA

Table VIII

Frequency of Isolates

N = 142

Organism	No	%
1. S.aureus	41	28.8
2. P. aeruginosa	17	11.9
3. K. pneumonia	20	14.0
4. K.oxytoca	7	4.0
5. Pr. vulgaris	4	2.8
6. Pr. mirabilis	11	7.7
7. CONS	10	7.0
8. E.coli	14	9.8
9. M. morganii	1	0.7
10. C.freundi	1	0.7
11. Strep pyogenes	8	5.6
12. Enterococci	4	2.8
13. Bacteroides	2	1.4
14. Peptostreptococcus	2	1.4

Total number of 142 isolates were obtained from the 100 cases of diabetic foot ulcer . S. aureus was found to be the most common gram positive

cocci (28.8%). Among gram negative bacilli Klebseilla spp were isolated in 27 cases (18%). Followed by P. aeruginosa in 17 cases. (11.9%) Anaerobic bacteria isolated from Grade III lesions were Bacteroides 2 (1.4%) and Peptostreptococcus 2 (1.4%)

Table IX

Poly VS Monomicrobial

N= 100

MONOMICROBIAL	POLYMICROBIAL	AVE: ORGANISM PER CASE
58	42	1.42

Among the 100 cases 58 foot ulcers were found to be monomicrobial and 42 were polymicrobial in nature. In an average 1.42 organisms per case was obtained.

Table X

Gram positive cocci Vs Gram negative bacilli

N = 142

Organism	No.	%
Gram positive cocci	65	45.1
Gram negative bacilli	77	54.3

Out of the 142 isolates obtained from the diabetic foot ulcers 65 (45.1%) cases were found to be Gram positive cocci and 77 (54.3%) were Gram negative bacilli.

Table XI

Antibiotic Sensitivity Pattern of Gram Positive Cocci

N = 63

	ANTIMICROBIAL AGENT	S. AUREUS N = 41 SEN. %		CONS N = 10 SEN%		STREP PYOGENES N = 8 SEN%		ENTEROCOCCI N = 4 SEN%	
1	AMOXYCILLIN	8	20%	7	70%	6	75%	2	50%
2	CIPRO	27	66%	9	90%	8	100%	4	100%
3	GENTAMICIN	25	61%	7	70%	7	87%	3	75%
4	CEPHALEXIN	20	49%	9	90%	6	75%	1	25%
5	CEFATAXIME	26	63%	10	100%	7	67%	3	75%
6	VANCOMYCIN	41	100%	10	100%	8	100%	4	100%
7	ERYTHROMYCIN	-	-	5	50%	5	62%	3	75%

Out of the 63 Gram positive isolates identified from the diabetic foot ulcers all were found to be sensitive to vancomycin. Among the isolates of S. aureus only 20% (8 cases) were sensitive to amoxicillin, 66% of S. aureus were sensitive to ciprofloxacin.

Table XII

Antibiotic Sessitivity Pattern of Gram Negative Bacilli

N=75

	ANTIMICROBIAL AGENT	K. pneumonia N = 20 Sensitive s%		K oxytoca N = 7 Sensitive s%		E coli N = 14 Sensitive s%		P aeruginosa N =17 Sensitive s%		Pr. Vulgaris N = 4 Sensitive s%		Pr. Mirabilis N=11 Sensitive s%	
1	AMIKACIN	11	55%	5	71%	7	50%	4	23%	2	50%	9	82%
2	CIPRO	4	20%	4	57%	4	28%	2	12%	3	75%	7	64%
3	CEFTRIOXON	9	45%	2	29%	6	43%	2	12%	2	50%	9	82%
4	CEFTAZDIME	12	65%	2	29%	4	28%	4	23%	2	50%	8	73%
5	IMIPENAM	18	90%	5	71%	17	78%	6	35%	3	75%	10	91%
6	PIP/ TAZO	11	55%	7	100%	13	93%	9	53%	4	100%	9	82%

Among the 75 isolates of Gram negative bacilli isolated from the diabetic foot ulcers Imipenam showed good sensitivity. Klebseilla Spp and Proteus spp showed 80-90% sensitivity towards Imipenam.

Screening for MRSA

Table XIII

Using oxacillin disc (1 ug)

N = 41

ZONE(mm)	NO: of isolates	%
> 14 MSSA	24	58.5
< 10 MRSA	17	41.5

All the 41 isolates of S. aureus were screened for methicillin resistance using oxacillin disc(1ug) and out of them 17(41.5%) were found to have a zone of inhibition less than 10mm for oxacillin (1ug) .

Table XIV

Using Cefoxitin disc (30ugs)

N = 41

ZONE(mm)	NO:of isolates	%
> 20 MSSA	23	56
< 19 MRSA	18	44

All the 41 isolates of S. aureus were then further confirmed for methicillin resistance using cefoxitin disc (30ug)

Table XV

PCR FOR mecA GENE IDENTIFICATION

N = 18

Total isolates of S.aureus	mec A present	mec A absent
18	18	nil

The 18 strains which showed resistance to Cefoxitin were subjected to mecA gene identification using the conventional PCR technique. All of the 18 isolates were positive for mecA gene.

SCREENING FOR ESBL

Table XVI

Resistance to Two 3rd Generation Cephalosporins

N = 75

ORGANISM	CEFTRIOXONE	CERTAZIDIME
K. pneumonia	11	10
K.oxytoca	5	5
E.coli	8	10
Pr. vulgaris	2	1
Pr. mirabilis	3	3
P. aeruginosa	15	15

Out of 75 isolates of Gram negative bacilli 40 were found to be resistant to two 3rd generation cephalosporins namely ceftrioxone and ceftazidime. The remaining were sensitive to at least one of the above 3rd generation cephalosporins.

Table XVII

Resistant gram negative bacilli

N = 40

Enterobactericeae	Pseudomonads
25	15

Out of the 40 isolates 25 were from the Enterobactericeae family and the remaining 15 were Psedomonads.

All the 25 isolates of Enterobactericeae were subjected to Double disc synergy test , Phenotypic confirmation test and MIC reduction test to detect

the presence of ESBL producers.The resistant Pseudomonads were subjected to Combined disc synergy test for the detection of Metallobetalactamases (MBL)

Table XVIII

Double Disc Synergy Test

N = 25

No. of Isolates	Positive	Negative
25	20	5

In the Double disc synergy method for identification of ESBL, cefataxime (30mcg) and amoxicillin/clavulanate (20ug/10ug) was used and out of the 25 isolates of enterobactericeae 20 were identified as ESBL producers.

ESBL Confirmatory test

TABLE XIX

Combined Disc Test

N = 25

No.of Isolates	**Positive**	**Negative**
25	22	3

Using the combined disc method as described in CLSI guidelines, out of the 25 isolates of enterobactericeae 22 were found to be ESBL producers.

Table XX

Minimum Inhibitory Concentration (MIC) of isolates to Ceftazidime (µg/ml)
N = 25

	Total	2	4	8	16	32	64	128	256	512	1024
Klebsiella pneumoniae	11	0	0	0	0	0	1	2	3	3	2
Klebsiella oxytoca	2	0	0	0	0	0	0	1	1	0	0
E.coli	7	0	0	0	0	0	1	1	2	3	0
P.mirabilis	4	0	0	0	0	0	0	0	0	1	3
P.vulgaris	1	0	0	0	0	0	0	0	0	0	1

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 64 µg/ml of agar to 1024 µg/ml of agar Reduction of (MIC) of isolates to Ceftazidime with 2µg/ml Clavulanic acid.

TABLE XXI

MIC Reduction in the presence of Ceftazidime with 2 µg/ml Clavulanic acid in Agar (N=25)

	Concentration of Ceftazidime with 2µg/mlClavulanic acid in Agar (µg/ml)									
	Total	0.5	1	2	4	8	16	32	64	128
Klebsiella pneumoniae	11	1	0	5	2	1	2	0	0	0
Klebsiella oxytoca	2	0	0	0	0	0	1	1	0	0
E.coli	7	0	0	0	1	2	3	1	0	0
P.mirabilis	4	0	0	0	0	2	0	0	0	2
P.vulgaris	1	0	0	0	0	0	0	0	1	0

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 0.5 µg/ml of agar to128 µg/ml of agar in the presence of clavulanic acid at a concentration of 2 µg/ml of agar showing 8 fold reductions in MIC.

Table XXII

ESBLS PRODUCERS

N = 75

Isolates of Gram negative bacilli	MIC REDUCTION Positive	COMBINED DISC Positive	ESBL%
75	22	22	29.3%

All the isolates of enterobacteriaceae screened as probable ESBL producers were tested with combined disc test and were subjected to MIC reduction test. Out of the 75 gram negative bacilli 22 were identified as ESBL producers in both methods.Hence the percentage of ESBL producers was 29.3%

TABLE XXIII

CTX – M Gene Identification in Klebseilla SPP.

N = 13

ORGANISM	NO: TESTED	CTX-M-GENE PRESENT	CTX-M GENE NEGATIVE	% POSITIVE
K.pneumonia	11	6	5	54.5
K.oxytoca	2	0	2	_

The CTX-gene responsible for the ESBL production was studied in the Klebseilla spp. Out of the 11 isolates of K.pneumonia 6(54.5%) were positive for the CTX-gene

TABLE XXIV

Screening for Metallobetalactamases Producers Among Pseudomonads

N = 15

Isolates	Positive	Negative	MBL%
15	14	1	93.3

P. aeruginosa resistant to imipenam were screened for MBL production by the combined disc method using 750ug of EDTA. Out of 15 isolates 14(93.3%) isolates were positive for the test .

Table XXV

OUTCOME OF THE STUDY

N= 62

OUTCOME	NO(%)	MRSA ISOLATE	ESBL ISOLATE	MDR PSUEDO	NON MRSA/ESBL
HEALED	18(29%)	1	2	2	13
FLAPSURGERY	14(22.5%)	3	3	1	7
REGULARDRESSING	24(38.7%)	8	3	4	9
AMPUTATION	6(9.6%)	3	2	0	1

Outcome was noted in 62 patients only as the remaining did not come for regular follow up. Out of the 62 patients healing was seen in 18(29%) patients and among them one (0.5%) was positive for MRSA,two(1.1%) had ESBL in the ulcers and two(1.1%) lodged MDR Pseudomonads and the remaining13(72%) did not harbour any resistant organism .Various flap surgeries depending upon the nature of the ulcer was done in 14(22.5%) of the patients.Out of them 50% had resistant organisms in the ulcers (MRSA-21.4%,ESBL-21.4% and MDR Pseudo-7%). 24(38.7%) patients were on regular dressing at the end of the period of study. Their ulcers did not heal even after regular dressings. Among the patients 15(24%) had resistant organisms in their ulcers (MRSA-33%, ESBL -12%, MDR Pseudo -16%,). Amputation was done in 6 (9.6%) of the patients out of which 3 (50%) showed MRSA, 2 (33%) showed ESBL and one patient did not have any resistant organism.

DISCUSSION

In the present study the total number of patients with diabetic foot ulcers were hundred. As per our study the total number of males were 53 and females were 47. The male to female ratio was 1:0.8. There were more number of males with diabetic foot ulcer than females in our study. This feature correlated with studies conducted by Dushyant Singh gaur et al[33],Sharma et al [35] and Lea Renina Ilanes [31].

The mean age of patients with diabetic foot ulcers in our study was 58.6.This observation was also made by Lea Renina et al.[31] and Gaur et al[33]. In our study most patients were in their 5th decade of life ,this was in contrary to other studies by Sharma et al and Llanes et al who reported diabetic foot ulcers to be more common in the 6Th decade.

Family history of Diabetes Mellitus was noted in 70% of the patients who took part in the study. Hence positive family history of Diabetes Mellitus was a significant factor in the development of foot ulcers in the patients. However family history of foot ulcers leading to foot ulcers in the sibling appeared only in eight patients. Poor glycaemia control (random blood glucose level >200mgs%) was found in 27% of the patients hence it is an important factor in wound healing. This finding was also noted by Sharma etal [35]

The most common risk factor for the development of Diabetic foot ulcers among the patients was observed as peripheral neuropathy which was

seen in 52% of the patients. Neuropathy predisposes a diabetic to unrecognized injury due to loss of sensation. Similar findings have also been reported by Llanes et al [31] and Sharma et al . [35] Patients also had co morbid conditions like hypertension, history of amputation and peripheral vascular disease indicating that ulcers develop from the interplay of multiple risk factors superimposed with infection.

Most of the patients in the present study presented with Wagner's grade II ulcers. Llanes et al [31] and Sharma et al [35] also made similar observation. This might be because of increased awareness among patients about foot care and regular screening done for foot ulcers at our hospital. Also because the patients in our study come to the department regularly for medicines and dressing of the ulcers.

The present study showed gram negative organisms (54.3%) to be more common in diabetic foot ulcers, this feature correlated with findings from studies done by Llanes et al [31], Shankar et al [27] and Bansal et al. However studies by Sharma et al [35] showed gram positive cocci to be more common among diabetic foot ulcers. S.aureus(28.8%) was found to be the most commonly isolated organism in our study ,this finding correlated with observations made by Llanes et al [31], Gaur et al [33] and Sharma et al [35] .In our study Klebseilla species(18%) was the most common gram negative organism which was contrary to other studies which have reported Pseudomonas to be the most common gram negative bacteria isolated from diabetic foot ulcers.

The polymicrobial nature of diabetic foot ulcers has been well documented by a number of authors like Llanes et al [31]. Sharma et al [35]. This feature was also noted in our study. The average organism per ulcers in our study showed 1.42 bacteria per case which was similar to findings by Vishwanathan et al(1.21) [36] and a little lower than Bansal et al(1.57). This might have been due to the fact that ulcers that we studied were mostly superficial ulcers and only few deeper ulcers were sampled.

All the gram positive organism isolated in our study were sensitive to vancomycin, this observation was also made by Sharma et al. Good sensitivity to ciprofloxacin was noted among the gram positive cocci(>90%) but for S.aureus (66%). Bansal et al also made similar observation. Sensitivity to 3rd generation cephalosporin was between 60 to 70%. Amoxycillin was the least sensitive drug among the gram positive cocci.

Among the gram negative isolates good sensitivity was seen for imipenem (>85%). Resistance to 3rd generation cephalosporin was high among the gram negative bacilli (60%) this feature was also noted by Gadepalli et al.[36] High rate of antibiotic resistance seen in our study may be due to the fact that ours is a tertiary care hospital with widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogen. Four anaerobes (2.8%) were isolated from grade III lesions.

Oxacillin resistance in isolates of S.aureus was used as a screening test for detection of MRSA in our studies but as the sensitivity and specificity of the test as reported by Swenson et al [(1)] was 86% and 74% respectively. We followed CLSI guidelines of using Cefoxitin (30ug) disc diffusion test for predicting the presence of the mecA gene which is responsible for methicillin resistance in S.aureus. Hence in our study MRSA identification using cefoxitin disc (30ug) correlated with all the mecA positive S.aureus. Studies done by Swenson et al [(1)] noted that this test had a sensitivity and specificity of 100%. Cefoxitin disc method has been shown to be superior to Oxacillin disc method for detection of MRSA by authors like Sandrin Roisin et al.[(16)].

The percentage of MRSA isolated in our study was 44% among the Staphylococcus aureus isolated which was closer to studies done by Tentoloisis al[(28)] (40%) and Lipsky et al[(29)]. However it was lower than studies done by Gadepalli (56%). Lipsky also noted that colonisation with MRSA was more common in Grade1 lesions. Gadepalli observed presence of MRSA doubles the healing time in diabetic foot ulcer. This observation was also made in our studies as 33% of the patients coming for dressing had MRSA.

In our study screening for ESBL producers in gram negative bacilli has been done using two 3rd generation cephalosporin as first line drugs in routine antibiogram as per CLSI guidelines. All isolates resistant to both the drugs were subjected to double disc synergy test but as this test has a lower sensitivity, phenotypic combined disc test was done. This method for identification of ESBL producers has been done in a numbers of studies like

Ami Variya et al.[39] In our study MIC for most of the ESBL producers was seen to be high (>64ug/ml) Similar feature was seen by Amit jain et al.[60].

CTX-M gene responsible for the production of betalactamase was identified in 6 out of 11 strains of Klebseilla spp, which was 54.5%, this feature correlated with studies done by Baby Padmini et al.[61]. They also found this gene to be most common in South India. In our study CTX-M positive Klebseilla were seen to have associated resistance to fluroquinalones, this finding was also observed by Baby Padmini et al. Overall the percentage of ESBL in our study was 39.3% among the enterobactericeae studied and 29.3% among the gram negative bacilli and this feature correlated with other studies done on diabetic foot ulcers by Ami Variya et al [39].

In our study the percentage of MBL producing Pseudomonads among the gram negative bacilli was 18.6% and this feature was also noted by Ami Varaiya et al (20%)[39].

The outcome of the ulcers was based on a number of factors. Healing was seen more in patients with grade I ulcers with good glycaemia control and those without resistant organism in their foot ulcer. Regular dressing for non healing ulcers was more common among patients with other co morbid conditions and also resistant organism in the ulcers which was seen in (24%) of the ulcers. Amputation was seen in 9.6% of the patients most of them had grade III ulcers and 50% of the ulcers showed MRSA suggesting the higher rates of amputation in patients with MRSA. This feature was also noted by Gadepalli et al[36] and Lipsky et al.[32].

SUMMARY

The present study showed gram negative organisms (54.3%) to be more common in diabetic foot ulcers. S.aureus (28.8%) was found to be the most commonly isolated organism in our study and Klebseilla species(18%) was the most common gram negative organism. P.aeruginosa was isolated from 12% of the cases. Four anaerobes (2.8%) were isolated from grade III lesions. The average organism per ulcers in our study showed 1.42 bacteria per case.

All the gram positive organism isolated in our study were sensitive to vancomycin. Good sensitivity to ciprofloxacin was noted among the gram positive cocci (>90%) but for S.aureus (66%).

Among the gram negative isolates good sensitivity was seen for imipenem (>85%). Resistance to 3rd generation cephalosporin was high among the gram negative bacilli (60%). The most common risk factor for the development of Diabetic foot ulcers among the patients was observed as peripheral neuropathy which was seen in 52% of the patients.

The percentage of MRSA in our study was noted to be 44%. MecA gene was positive in 18 strains which was 100% of the isolates of Staphylococcus aureus tested using conventional PCR technique.

ESBL producing isolates were selected based on their resistance to two 3rd generation cephalosporin. The presence of the gene responsible for the production of ESBL namely CTX-M gene was present in 6 out of 13 isolates of Klebseilla spp.which was 54.5%. In our study the percentage of ESBL producers was 29.3%.

CONCLUSION

The foot problem in diabetic patients is multifaceted and three great pathologies come together in its development namely neuropathy, ischaemia and infection. Our study addresses infection in the foot ulcers of patients attending the Institute of Diabetology at Kilpauk Medical College &,Hospital Chennai.

- We found the diabetic foot ulcers to be polymicrobial in nature and Gram negative bacteria(54.3%) to be more common then gram positive bacteria (45.1%).However Staphylococcus aureus(28.8%) was seen as the most common bacterial pathogen followed by Klebseilla spp(18%) and Pseudomonads.(12%)

- Our studies showed the foot ulcers to be higher in patients with diabetes in the family and who had co morbid risk factor of which neuropathy was the most common. Poor glycaemia control also appeared as a significant factor for healing of the ulcer.

- On analyzing the antibiotic sensitivity pattern of the isolates we observed an alarming number of multidrug resistant organisms in the diabetic foot ulcers . 44% of S. aureus isolated were MRSA, 39.3% of the enterobactericeae were ESBL producers and 82.3% of Pseudomonads were MBL producers. .

- The high rates of antibiotic resistance observed in the present study may be due to the fact that ours is a tertiary care hospital with widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogens.

- The outcome of the patients depended on a number of factors like grade of the ulcer, associated co-morbid conditions and the presence of resistant pathogen.

- Our study showed that resistant isolates to be present in 54 cases of the diabetic foot ulcers. MRSA was seen in 18 cases, ESBL in 22 cases and MBL producing Pseudomonads in 14 cases.

- Our study showed that amputation was more common in ulcers which had MRSA(50%).Wound healing was delayed in patients with resistant bacteria-33% MRSA, 12% ESBL and 16% Multidrug resistant Pseudomonads. Healing of wound was maximum in grade I ulcers and those ulcers which did not show the presence of resistant bacteria namely 72% of the ulcers.

BIBLIOGRAPHY

1. Swenson J. M,Results of Disk Diffution testing with cefoxitincorralate with mecA in Staphyloccus spp.J.Clin.Microbiol.Aug2005 pg 3818 - 23

2. Swyth R.W.Mannitol salt agar –cefoitin combination as a sreening medium for Methicillin resistant staphyloccus aureus, J.Clin.Microbiol.Aug2005 pg 3797 - 99

3. Hayden M.K.,Development of Daptomycin resistance in Vivo in Methicillin Resistant Staphyloccus aureus, J.Clin.Microbiol.Oct 2005pg 5285 - 87

4. Rapid Differentiation of Methicillin susceptible Staphylococcus aureus from blood cultures by use of a direct cefoxitin disk diffusion method. J.Clin.Microbiol.Nov2008pg 3836 - 38

5. Arakere.G Genotyping of Methicillin resistant staphylococcus aureus Strains from two hospitals in Bangalore south India. J.Clin.Microbiol. July2005pg 3198 - 3202

6. Mehta.M.S Identification of Staphylococcus species directly from positive blood culture broth by use of molecular and conventional method. .J.Clin.Microbiol.Apr2009 pg 1082 - 86

7. Bocher .S,Staphyloccus lugdenensis, a common cause of skin and soft tissue infections in the community. J.Clin.Microbiol.Apr2009. 946 - 50

8. Ellis.M, Presence and Molecular epidemiology of Virulence factors in Methicillin resistant Staphylococcus aureus strains colonising and infecting soldiers. J.Clin.Microbiol.Apr2009 J.Clin.Microbiol. Apr2009 pg940 - 45

9. Flayhart.D.Multicenter evaluation of BBL CHROMagar MRSA medium for direct detection of Methicillin resistant Staphylococcus aureus from surveillance culture of the anterior nares J.Clin.Microbiol.Nov2005 pg 5536 - 40

10. Nagaoka T, Use of Three dimensional Microarray system for the detection of Levofloxicin resistance and the mecA gene in the Staphylococcus aureus. J.Clin.Microbiol.Oct2005 pg 5187 - 94

11. Dasjardins.M Evaluation of the IDI-MRSA for the detection of Methicillin resistant Staphylococcus aureus from nasal and rectal specimens pooled in a selective broth. J.Clin.Microbiol.Apr2006 1219- 23

12. Layer.F.Heterogeneity of Methicillin susceptible Staphylococcus aureus strains at a German University implicates the circulating strain pool as a potential source of Emerging Methicillin resistant S.aureus clones. J.Clin.Microbiol.June2006 2179 - 85

13. Ari Robiesek, Prediction of Methhicillin resistant Staphylococcus aureus invovement in diease sites by concomitant nasal sampling J.Clin.Microbiol.Feb2008 pg588 - 92

14. Feil.E.J, Rapid detection of the pandamic Methicillin resistant Staphylococcus aureus cloneST239, adominant strain in Asian hospitals. J.Clin.Microbiol.Apr2006 pg.1520 - 22

15. Goldmeyer.J, Identification of Staphylococcus aureus and determination of methicillin resistance directly from positive blood cultures by Isothermal Amplification and a disposible detection device J.Clin.Microbiol.Apr2008pg 1534 – 36

16. Performance of CHROM Agar and Oxacillin Resistant Screening Agar Base Media for Detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) from Chronic Wound. Modern applied science vol.3, No.5, May 2009.

17. Detection of Methicillin Resistant Staphylococci by Using Polymerase Chain Reaction. Journal of Clinical Microbiology, vol.30, No.7, July 1992, p. 1685-1691.

18. Gupta.K. Trends in prescribing B eta lactam antibiotics for treatment of community associatedMethicillin resistant Staphylococcus aureus infections, J.Clin.Microbiol.Dec 2007pg 3930 - 34

19. Tenover F.C Multiple locus Variable number tantam repeat assay analysis of methicillin resistant staphylococcus aureus strains, J.Clin.Microbiol.July2007pg 2215 – 19.

20. Zhang S X.Comparision of two version of the IDIMRSA assay using charcoal swabs for prospective nasal and non nasal surveillance samples. J.Clin.Microbiol.July2007pg 2278 - 80

21. Moroney.S.M Staphylococcus cassette chromosome mec and panton Valentine leukocidin charecterisation of Mrsa clones. J.Clin.Microbiol.May2007 pg 1019 - 21

22. GoldmeyerJ. Identification of Staphylococcus aureus and determination of methicillin resistance directly from positive blood culture by isothermal amplification and a diaposible detection device. J.Clin.Microbiol.Apr2008

23. Roisin.S Evaluation of New Vitek card and disk diffution method for determining susceptibility of Staphylococcus aureus to oxacillin J.Clin.Microbiol.Aug2008

24. Mohanasundaram.K.M Comparision of phenotypic versus genotypic methods in the detection of Methicillin resistance in Staphylococcus aureus. Indian.J.Med .Res127,Jan2008.78 - 84

25. Summaiya.M.Study of antibiotic sensitivity pattern of Methicillin resistant Staphylococcus aureus.Indian journal of critical care medicine.Vol.11 ,2007pg 99 - 101

26. Ekta Bansal,spectrum of microbial flora in Diabetic foot ulcer.Indian J.Pathol. Microbial.2008:51pg 204 - 07

27. Shankar.E.Bacterial etiology of diabetic foot infections in South India.European journal of internal medicine.Vol16(8)567 - 70

28. Armstrong DG, Lipsky BA. Diabetic foot infections: stepwise medical and surgical management. Int Wound J 2004;1:123—132.

29. H. SALIMNIA Detection of Oxacillin Resistance in *Staphylococcus aureus*: Comparison of Phoenix Oxacillin and Cefoxitin MICs,

30. MicroScan Oxacillin MIC, Oxacillin and Cefoxitin Disk Diffusion, and *mec*A Gene Detection.Antimicrobial agents and Chemotherapy 2005

31. Gadapalli R. Aclinicomicrobiological study of diabetic foot ulcers in a Indian tertiary care hospital.Diabetas Care Vol29Aug2006pg 1727 – 32.

32. Llanes L.R.Clinical micrabiological profile and outcome of diabetic patients with foot ulcers admitted at the Quirino Memorial Medical center.Diabetes medicine2000 17

33. Citron D.M. Bacterialogy of moderate to severe diabetic foot infections and in vitro activity of antimicrobial agents J.Clin.Microbiol.Sep2007pg 2819 - 28

34. Gaur.D.S.Diabetic foot in Uttaranchal .JK.Medicine vol 9 mar2007.pg 18 - 20

35. Bowler P.G.archaBacterial growth guidelines.Ostomy wound management vol49(1)Jan 2003.123 - 31

36. Sharma VK.Common pathogens isolated in diabetic foot ulcers in Bir hospital.Kathmandu univ.Medical journal 2006Vol4(3)pg – 295 - 301

37. Vishwanathan V. Prevelence of pathogens in diabetic foot infections in South Indian Type 2 diabetic patients.J.Assoc. Physicians India.2002aug50

38. Greenhalgh.d. Wound healing and diabetes mellitus, Clinics in plastic surgery30 (2003) – 45 pg – 37 - 45

39. Ami Varaiya, Incidence of metallobetalactamase producing P.aeruginosa in diabetes and cancer patients. Indian J.Pathol. Microbial.2008:51(2)-pg- 200 - 203

40. Ami Varaiya Extended spectrum beta lactamase producing E.coli and K.pneumoniae in diabetic foot infections. Indian J.Pathol. Microbial.2008:51(3) pg 370 - 372

41. Moller.KK. Distribution , Organisation and Ecology of Bacteria in chronic wounds J.Clin.Microbiol.Aug2008.pg 2717 - 22

42. Junyoung Kim1, Rapid Detection of Extended Spectrum β-Lactamase (ESBL) for Enterobacteriaceae by use of a Multiplex PCR-based Method. Infection and Chemotherapy : Vol.41, No.3, 2009

43. Kingsley,Sequence analysis of blaCTX-M-28 an ESBL responsible for third generation cephalosporin resistance in Enterobactericeae for the first time in India . Indian J.Pathol. Microbial.2008:51(2)

44. Pitout.J.D. Detection of P.aueruginosa producing MetalloBeta lactamase s in a Large centralized laboratory. J.Clin.Microbiol.July2005 3129 - 35

45. Marchiaro. Sensitive EDTA based microbiological assays for detection of metalloBetalactamases in non fermentative gram negative bacteria J.Clin.Microbiol.Nov2005pg – 5648 - 52

46. Behera, An Evaluation of four different phenotypic techniques for detection of metallo-β-Lactamase producing pseudomonas Aeruginosa Indian Journal of Medical Microbiology, (2008) 26(3): 233-37.

47. Varaiya. A Incidence of Carbapenem-Resistant Pseudomonas Aeruginosa in Diabetic and Cancer Patients. Indian Journal of Medical Microbiology, (2008) 26(3): 238-40.

48. Irfan, S Metallo-β-Lactamase Producing clinical isolates of Acinetobacter species and pseudomonas Aeruginosa from Intensive care unit patients of a tertiary care hospital. Indian Journal of Medical Microbiology, (2008) 26(3): 243-45.

49. Koneman's Color Atlas and Textbook of Diagnostic Microbiology 6th Edition 2006 Lippincott Williams & Wilkins,pg 974 - 1014

50. Mackie and McCartney Practical Medical Microbiology, 14th edition, 2006 Churchill Livingstone pp. 166-169

51. Manual of Clinical Microbiology by Patrick R. Murray, 8th edition 1083-1085.

52. Topley & Wilson's Microbiology & Microbial Infections 10th Edition Bacteriology by S. Peter Borriello, Patrick R. Murray and Guido Funke. Hodder Arnold Publishers.

53. PaganiLNosocomial outbreak caused by multidrug reristant p.Aeruginosa producing IMP-13 metallobetalactamases. J.Clin.Microbiol.Aug2005pg -3824 - 28

54. Yohei Doi, Simple disk based method for the detection of K.pneumoniae carbapenamase Type betalactamases by use of a boronic acid compound J.Clin.Microbiol.Dec2008.pg 4083 –86

55. Samra Z.Evaluation of CHROMagar KPC for Rapid detection of carbapenam resistant Enterobacteriaceae. J.Clin.Microbiol.Sep2008 pg 3110 - 11

56. Mendes.R.E, Rapid detection and identification of metallobetalactmases-encoding genes by multiplex rel time PCRassay and melt curve analysis. J.Clin.Microbiol.Feb2007pg – 544 - 547

57. Ting-ting Qu .Evaluation of the phynotypic tests for detection of metallobetalactamase producing P.aeruginosa strains in China. J.Clin.Microbiol.Nov2009. pg 1136 - 42

58. Bjorn.A. Metallobetalactamse or Extended-spectrum betalactamases :a wolf in the sheep's clothing. J.Clin.Microbiol.June2007 - 2034 - 36

59. Youri Glupezynski.Evaluation of a new selective Chromogenic ager medium for the detection of extended betalactamases producing Enterobacterceae. J.Clin.Microbiol.Feb2007 pg -501 - 505

60. Tofteland.S.Effects of phenotype and genotype on the methods for detection of extended spectrum betalactamase- producing clinical isolatesof E.coli and K.pnemoniae in Norway. .Clin.Microbiol.Jan2007 pg -199 -205

61. Amita Jain.TEM & SHV genes in the extended spectrum betalactamase producing Klebseilla species & their antimicrobial resiatance pattern. J.Med .Res128,Dec2008. Pg – 759 - 764.

62. Baby Padmini .Detection of Enterobactericeae producing CTX-M extended spectrum betalactamases from a tertiay care hospital in South India Indian journal of medical microbiology (20080 26(2).163 - 6

63. Shazi Shakil, Asad U Khan Infected foot ulcers in male and female diabetic patients: a clinico-bioinformative study. Annals of Clinical Microbiology and Antimicrobials 2010, 9:2

64. Black A. AmpC disk test for the detection of plasmid mediated AmpC betalactamases in Enterobactericeae lacking cchromosomal AmpC Betalactamases. J.Clin.Microbiol.July2005 pg 3110 - 3113

65. Coudron P.E Inhibitor based methods for detection of plasmid mediated AmpC betalactamases in Klebseilla spp. E.coli and Proteus mirabilis. J.Clin.Microbiol.Aug2005,pg 4163 - 67

66. KIM, YUN-TAE, TAE-UN KIM, AND HYUNG-SUK BAIKCharacterization of Extended Spectrum β-Lactamase Genotype TEM, SHV,and CTX-M Producing Klebsiella pneumoniae Isolated from Clinical Specimens in Korea. J. Microbiol. Biotechnol. (2006), 16(6), 889–895.

67. bell. J.M. Prevelence and significance of Negative Extended spectrum betalactamase confirrmation test after a positive ESBL screening test result for isolates of E.coli and K.pneumoniae. J.Clin.Microbiol.May2007pg – 1478 - 82

68. Sinha P. Prevelence of ESBL producers among E.coli isolates in a tertiar care hospital in jaipur. Indian J.Pathol. Microbial.2008:51(3)pg 367 - 69

69. Sridhar Rao.P.N.Detection of ESBL from clinical isolates in Davengere. Indian J.Pathol. Microbial.2008:51(4) pg- 497 - 99

70. Karcher.Microbiology and treatment of Diabetic foot infections pg210 - 217

71. Clinical Laboratory Standards Institute (CLSI) 2006, Wayne, Pa.

APPENDIX

1. MacConkey agar:-

This is a useful medium for the cultivation of enterobacteriaceae. It contains a bile salt to inhibit non-intestinal bacteria and lactose with neutral red to distinguish the lactose-fermenting coli forms from the lactose –non-fermenting salmonella and shigella groups. The concentration of sodium taurocholate may be reduced to suit less tolerant organisms. The omission of sodium chloride from the medium prevents the spreading of Proteus colonies.

Peptone	20 g
Sodium taurocholate, commercial	5 g
Water	1 litre
Agar	20 g
Neutral red solution, 2% in 50% ethanol	3.5 ml
Lactose, 10% aqueous solution	100 ml

Dissolve the peptone and taurocholate (bile salt) in the water by heating. Add the agar and dissolve it in the steamer or autoclave. If necessary, clear by filtration. Adjust the pH to 7.5. Add the lactose and the neutral red, which should be well shaken before use, and mix. Heat in the autoclave with 'free steam' (c. 100^{o} C) for 1hr., then at 115^{o} C for 15 min. Pour plates.

2. Nutrient agar:-

	Gm/L
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	5.00
Agar	15.00

Dissolve the contents in water and mix by heating Autoclave at 121^{o} C for 15 minutes. Adjust pH to 7.4 $\pm$ 0.2. Pour 20-25 ml of 9 cm dia. Petridishes to give 4 mm thickness.

3. Blood agar:-

Sterile sheep blood	50 ml
Peptone	10 g
Beef extract	3g
Sodium chloride	5 g
Distilled water	1000 ml

Autoclave the nutrient agar base at 121^{o} C for 15 minutes and blood with sterile precautions and distribute in Petri dishes.

For Anaerobic culture blood agar was supplemented with vitK(10ug/ml) and hemin(5ug/ml).

4. Muller Hinton agar:-

Beef infusion	300	ml
Casein Hydrolysate	17	gm
Starch	1.5	gm
Agar	10	gm
Distilled water	1000	ml

Emulsify the starch in a small amount of cold water, pour into the beef infusion and add the casein-Hydrolysate and the agar. Make up the volume to 1000 ml (1 litre) with distilled water. Dissolve the constituents by heating gently at 100^{o} C with agitation.

Adjust the pH to 7.4. Dispense in screw-capped bottles and sterilize by autoclaving at 121 ^{o} C for 20 minutes. 20 to 25 ml of it is poured into petridishes of 9 cm diameter to give a thickness of 4mm.

5. McFarland's Turbidity Standard for inoculum preparation

A Barium sulphate 0.5 McFarland standards was prepared as follows

1. A 0.5 ml of 0.048mol/L of Barium chloride was added to 99.5 ml of 0.18 mol/L of H2SO4 with constant stirring to maintain a suspension.
2. Correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance of 625nm should be 0.08 to0.10 for the 0.5 McFarland standards.
3. The Barium sulphate suspension was transferred in 4-6 ml to a screw capped tube of the same size as those used in growing or diluting the bacterial inoculam.
4. These tubes were tightly sealed and stored in the dark at room temperature.
5. The Barium sulphate turbidity standard was vigorously agitated before each use and inspected for a uniform turbid appearance.

Differentiating Characters of isolates commonly seen in diabetic foot ulcer

For Gram positive cocci (GPC)

Gram stain	Catalase	oxidase	Hemolysis	Coagulas	MSA	Mannitol	BSA	Bacitraci	organism
Cocci in clusters	+	-	+/-	+	+	+	-	R	Staphylococcus aureus
Cocci in clusters	+	-	+/-	-	-	-	-	R	CONS
Cocci in Chains/pair	-	-	+	-	-	-	-	S	Streptococcus pyogenes
Cocci in pairs	-	-	+/-	-	-	-	+	R	Enterococci

Differentiating Characters of isolates commonly seen in diabetic foot ulcer For Gram negative Bacteria[GNB}

Organism	Motility	Oxidase	Catalase	MAC	Indole	TSI	PPA	NO_2 REDUCTION	MR	VP	Citrate	urease	Glucose	Lactose	Sucrose	Maltose	Mannose
Escherichia coli	+	-	+	LF	+	A/A	NA	+	+	-	Not utilised	-	+	+	+	+	+
Klebsiella Pneumonia	-	-	+	LF	-	A/A	NA	+	-	+	utilised	+	+	+	+	+	+
Klebsiella oxytoca	-	-	+	LF	+	A/A	NA	+	+	-	utilised	+	+	+	+	+	+
Proteus. species	+	-	+	NLF	±	K/A with H2S	G	+	+	-	+	+	+	-	+	-	-
Pseudomonas .aeruginosa	+	+	+	NLF	+	K/K	NA	+			utilised		-	-	-	±	-
M.morganii	+	-	+	NLF	+	K/A	G	+	+	-	Not utilised	+	+	-	-	-	-
C.freundi	+	-	+	NLF	+	A/A	NA	+	+	-	utilised	+/-	+	+	+	+	+

Note: A/A = Acid slant / Acid butt
K/A = Alkali slant / Acid butt
K/K = Alkali slant / Alkalibutt

\+ = Positive,
\- = Negative
NA = Not applicable

ZONE SIZE INTERPRETATIVE CHART IN ACCORDING TO CLSI

Kirby-Bauer Chart

Sl. No.	Drug	Disk Content mcg	Resistant mm or less	Intermediate mm	Sensitive mm or more
1	Amoxycillin	10 mcg	14 mm	15-16 mm	17 mm
2	Co-trimoxazole	1.25+3.75	10	11-15	16
3	Cephalexin	30	14	15-17	18
4	Norfloxacin	10	12	13-16	17
5	Ciprofloxacin	5	15	16-20	21
6	Gentamycin	10	12	13-14	15
7	Cefotaxime	30	14	15-22	23
8	Ceftriaxone	30	13	14-20	21
9	Ceftazidime	30	14	15-17	18
10	Amikacin	30	14	15-16	17
11	Imipenam	10□g	$\leq$ 13	14-15	$\geq$ 16

DIABETIC FOOT ULCER

SPECIMEN COLLECTION

1. **Sterile Swab**
2. **RCM Broth**

SPECIMEN PROCESSING

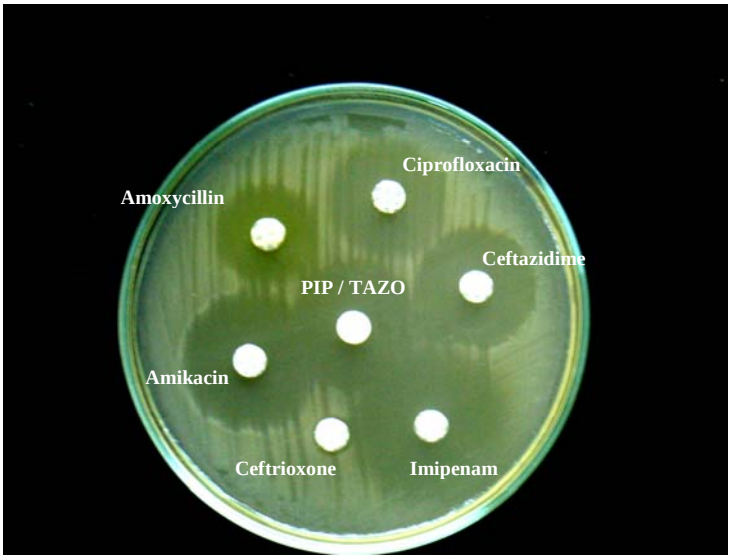
INOCULATION INTO PRIMARY PLATES

1 – Nutrient Agar Plate

2 – Mac Conkey Agar Plate

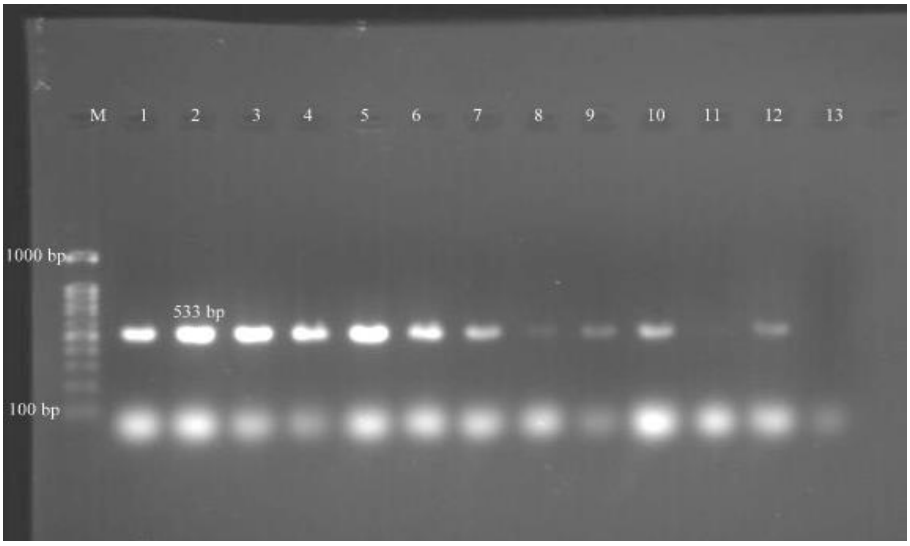
3 – Blood Agar Plate

ANTIBIOGRAM



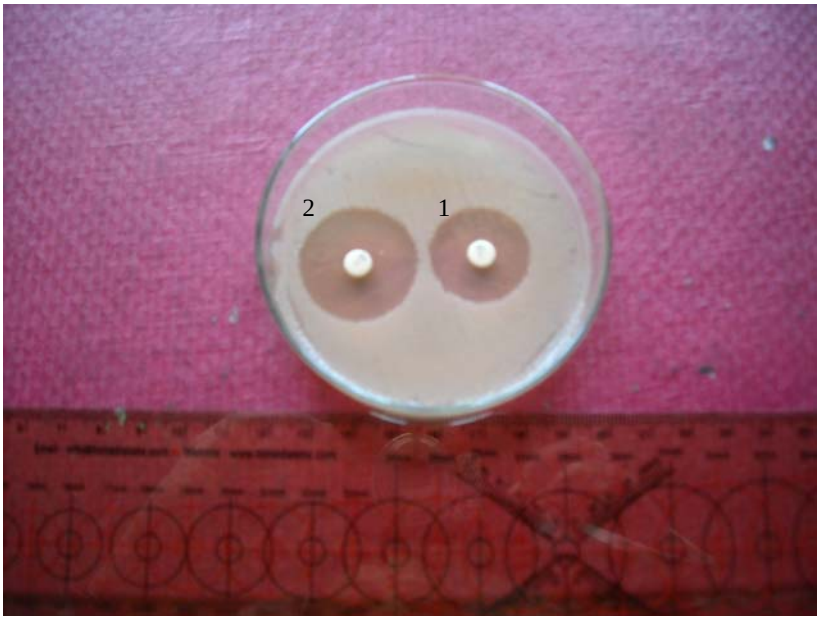
DETECTION OF MRSA USING CEFOXITIN DISC

DETECTION OF mecA GENE IN MRSA



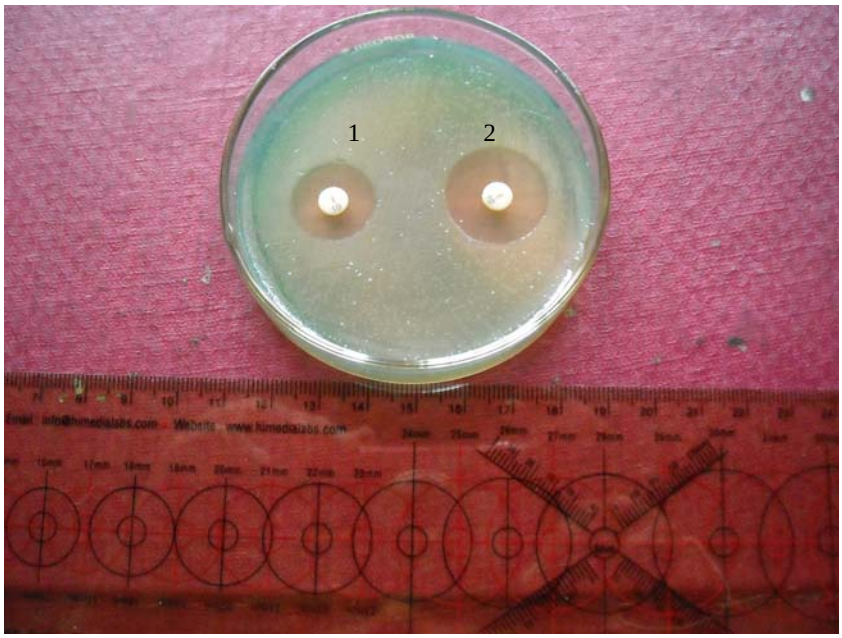
Molecular Marker	**-**	**100bp**
Lane 1 TO 11	**-**	**Sample**
Lane 12	**-**	**Positive Control**
Lane 13	**-**	**Negative Control**
Gel Percentage	**-**	**1.2%**

COMBINED DISC SYNERGY TEST FOR ESBL



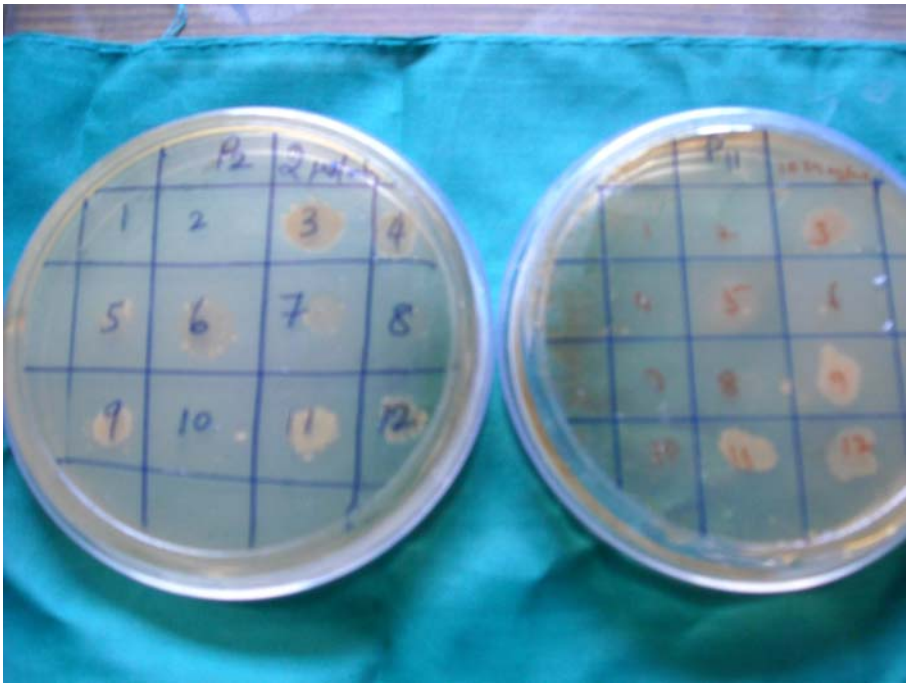
1. **Cefatazidime 30 μgs.**
2. **Cefatazidime 30 μgs + Clavulanic acid 10 μgs.**

COMBINED DISC SYNERGY TEST FOR MBL PRODUCERS

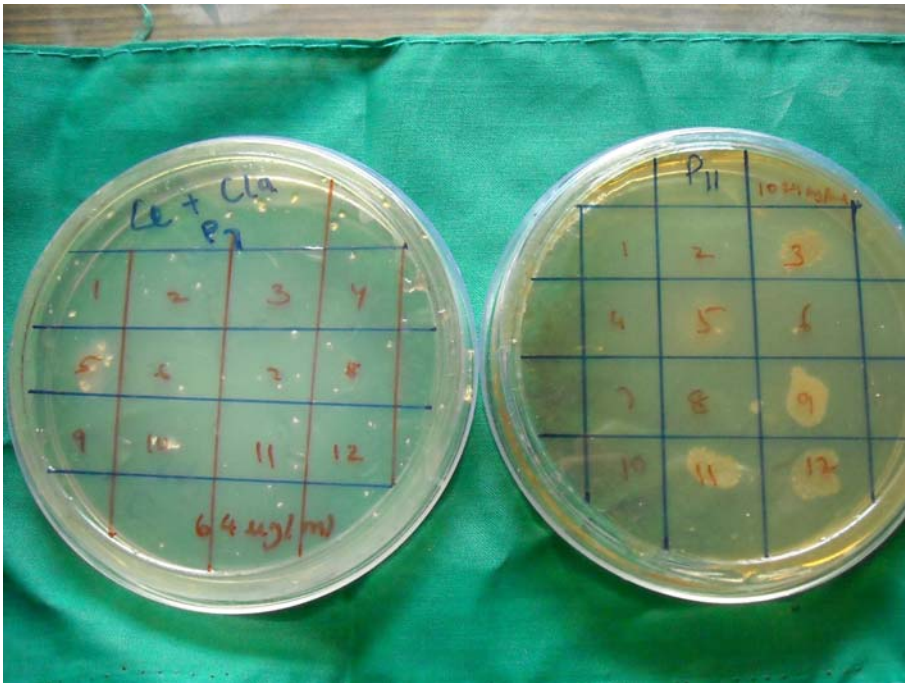


1. **Imipenam 10 μg**
2. **Imipenam 10 μg + EDTA (750 μg)**

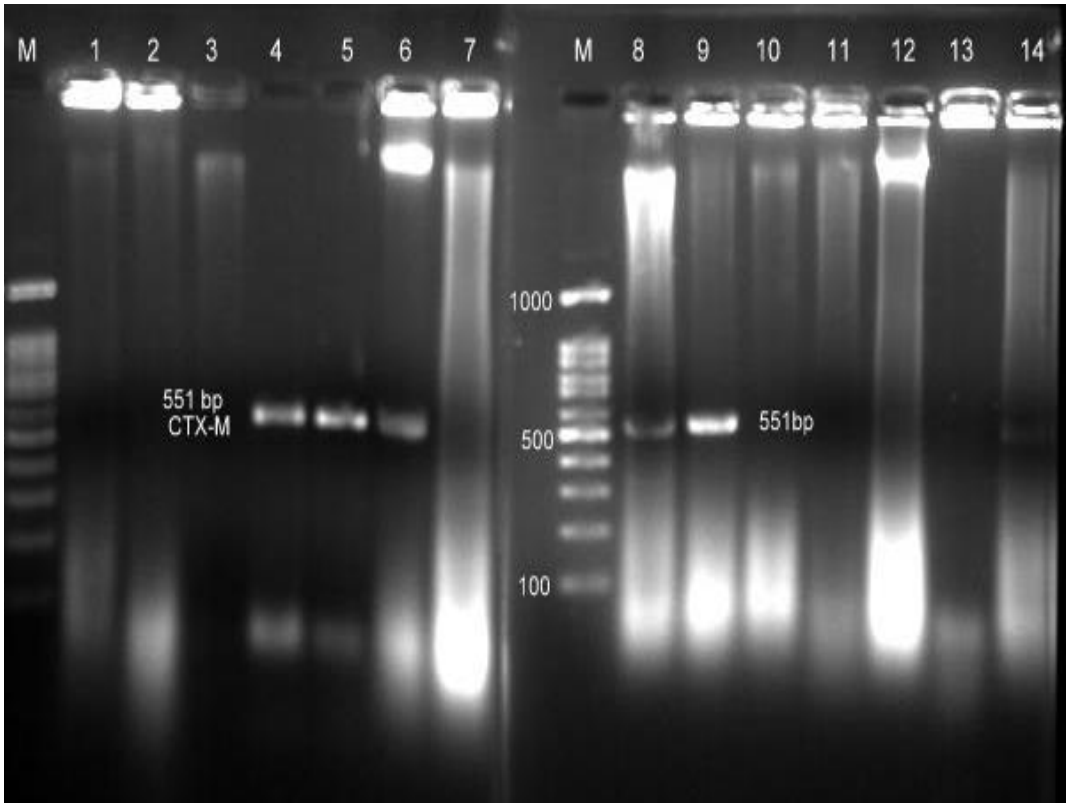
MIC OF CEFTAZIDIME



REDUCTION OF MIC OF CEFTAZIDIME IN THE PRESENCE OF CLAVULANIC ACID (2μ/ML)



DETECTION OF CTX – M GENE IN KLEBSEILLA SPP.



Molecular Marker	**-**	**100bp**
Lane 1 TO 8, 11-13	**-**	**Sample**
Lane 9	**-**	**Positive Control**
Lane 10	**-**	**Negative Control**
Gel Percentage	**-**	**1.2%**

Chart 1
Demography of Patients

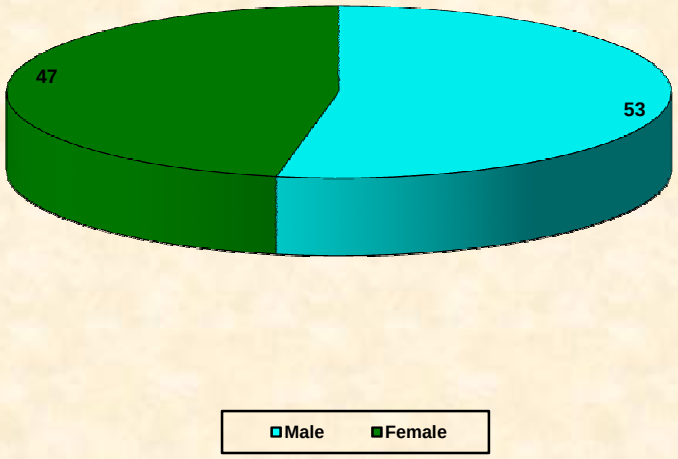


Chart 2
Age Distribution

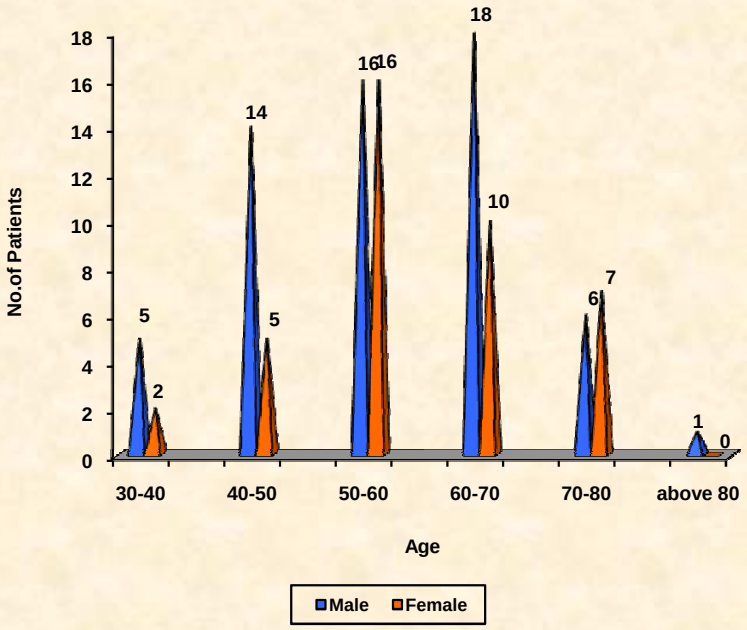


Chart 3
Risk Factors

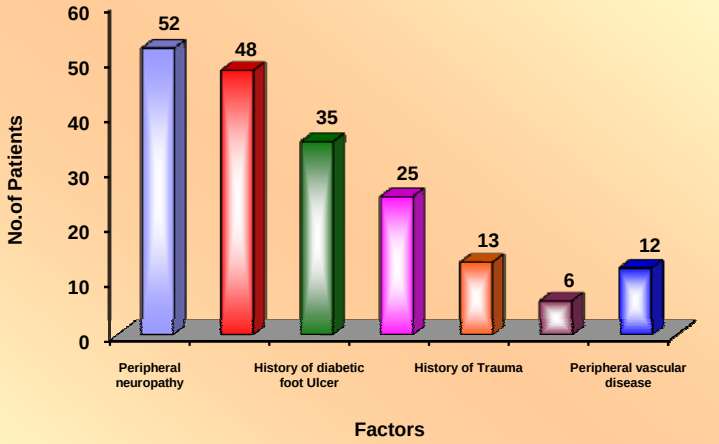


Chart 4
Wagnar's Classification of Diabetic foot ulcers

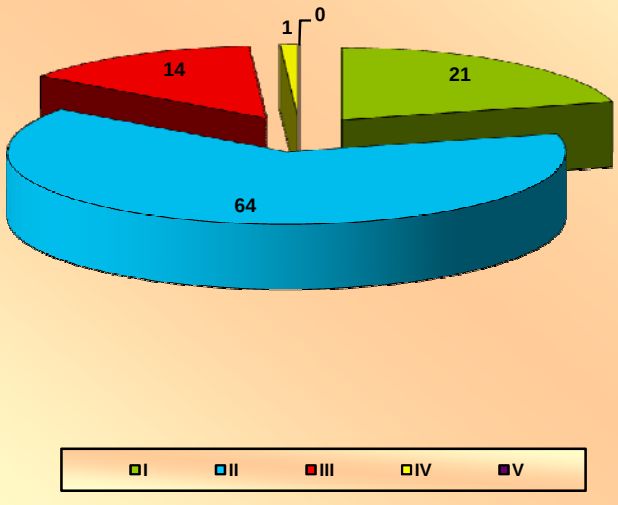


Chart 6
Antibiotic Sensitivity Pattern of Gram Positive Cocci

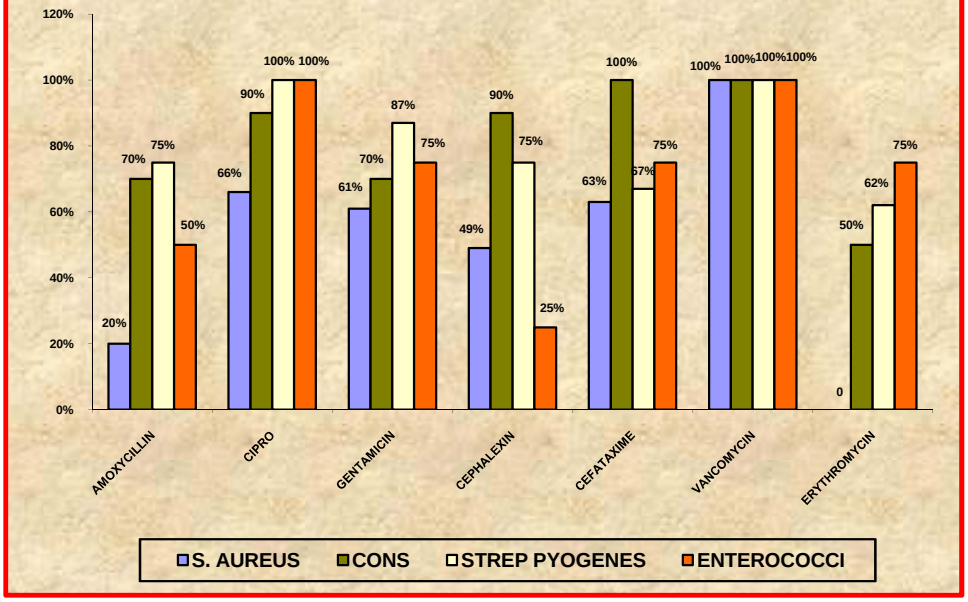


Chart 7
The Antibiotic Sensitivity pattern of gram negative bacilli

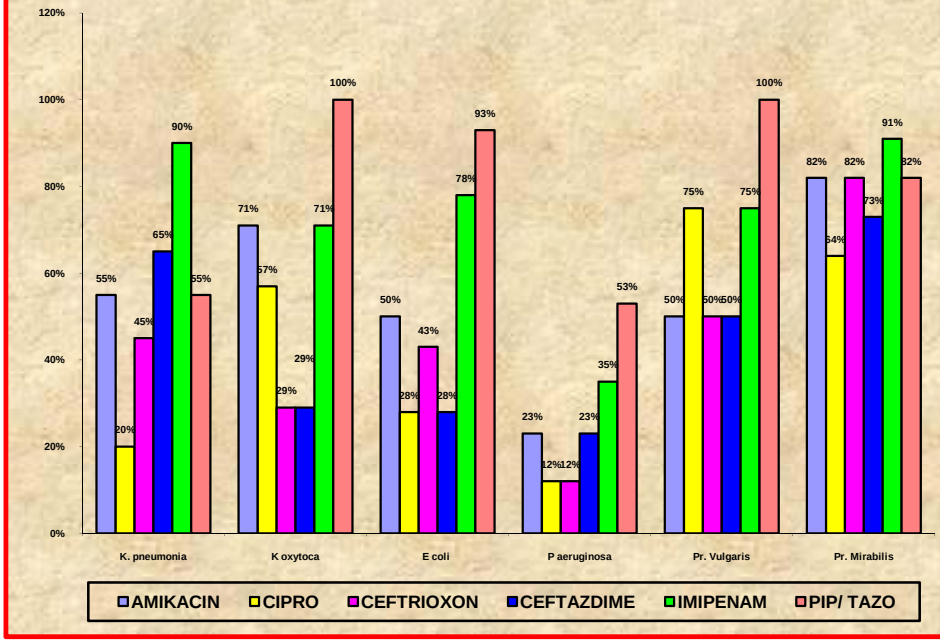


Chart 8
Percentage of MRSA Among S.aureus

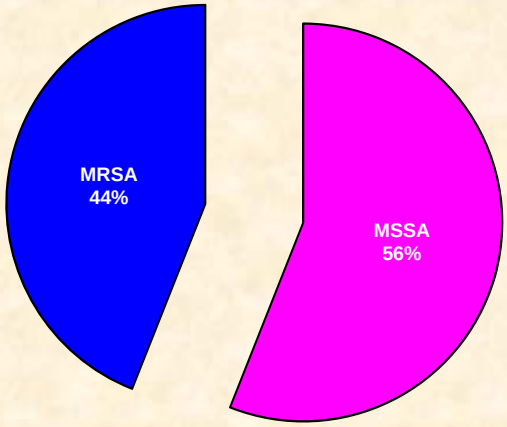
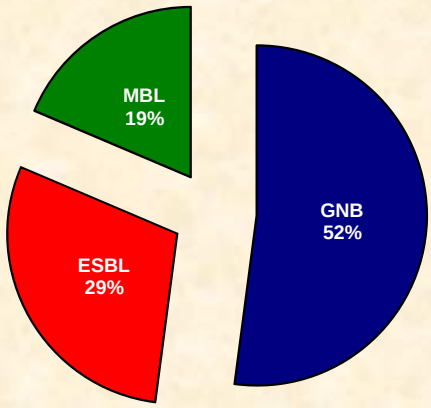


Chart 9
Percentage of ESBL and MBL among GNB



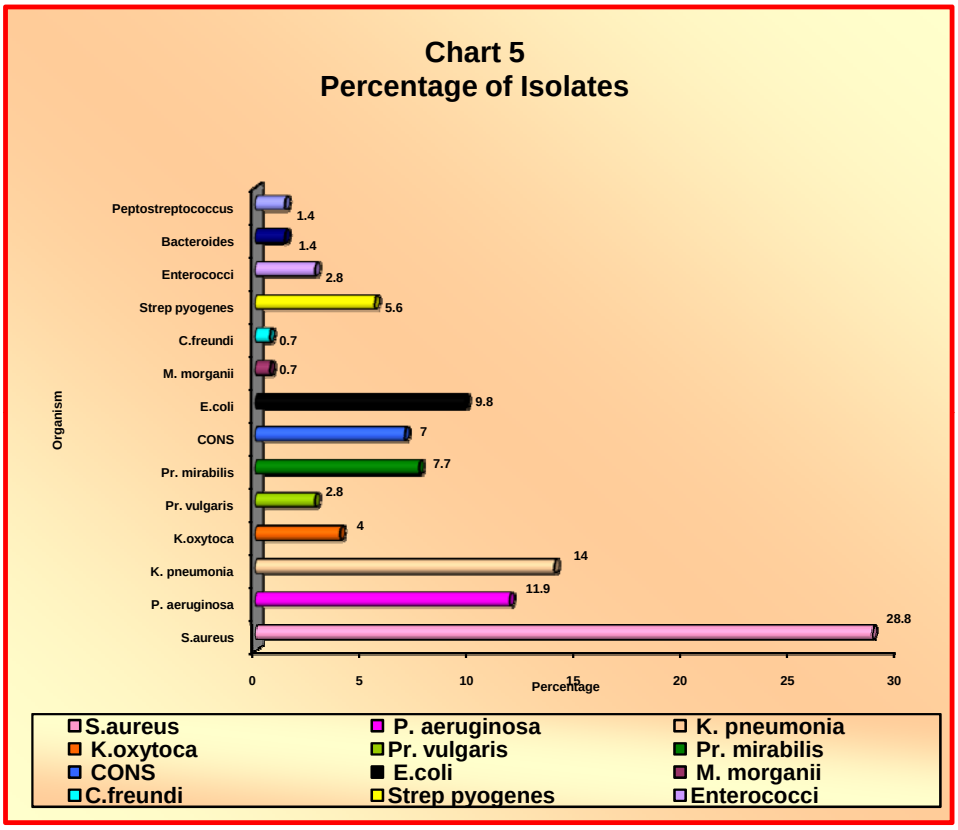


Chart 5
Percentage of Isolates
Organism
Peptostreptococcus
Bacteroides
Enterococci
Strep pyogenes
C.freundi
M. morganii
E.coli
CONS
Pr. mirabilis
Pr. vulgaris
K.oxytoca
K. pneumonia
P. aeruginosa
S.aureus
1.4
1.4
2.8
5.6
0.7
0.7
9.8
7
7.7
2.8
4
14
11.9
28.8
0
5
10
15
20
25
30
Percentage
S.aureus
P. aeruginosa
K. pneumonia
K.oxytoca
Pr. vulgaris
Pr. mirabilis
CONS
E.coli
M. morganii
C.freundi
Strep pyogenes
Enterococci

MASTER CHART

ID.NO	D.NO	AGE	SEX	DURATION	FAMILY HISTORY	RISK FACTOR	H/O HOSPITAL	ULCER SITE	GRADE	BLOOD GLUCOSE LEVEL	ISOLATE	P/M	MRSA	ESBL	MDR PSEUDO	OUTCOME
18/08	1/8	58	M	1 Year	NO	HT	NO	Rt dorsom	II	186%	S.aureus ,.Koxytoca	P	NO	NO	NO	HEALED
67/08	2/8	64	F	5 Months	NO	HT	NO	Rt bigtoe	II	298 mg%	s.aureus	M	NO	NO	NO	NO FOLLOW UP
56/08	3/8	51	M	1Year	NO	_	NO	Rt dorsom	II	260 mg%	S.aureus,Pseudo	P	NO	NO	YES	HEALED
459/06	4/8	65	M	2 Years	Yes	H/o previous ulcer	NO	Rt dorsom	III	99 mg%	Kp,Pv	P	NO	YES	NO	AMPUTATION
48/08	5/8	68	M	10 Years	Yes	PN, Retinopathi	NO	Lt dorsom	II	95%	S.aureus	M	NO	NO	NO	HEALED
6/7	6/8	58	F	22 Years	NO	HT, AMP	NO	Rt dorsom	II	154%	S.aureus,Pseudo	P	YES	NO	YES	FLAP SURGERY
49/08	7/8	71	M	8Years	Yes	HT, PN, AMP	NO	Rt G-toe	II	83 mg%	Kp,Pv	P	NO	YES	NO	NO FOLLOW UP
296/07	8/8	56	F	7 Years	NO	H/o previous ulcer	NO	Rt G-toe	II	187 mg%	S.aureus	M	YES	NO	NO	HEALED
241/07	9/8	39	F	2 Years	Yes	PN	NO	Rt dorsom	II	123 mg%	P.mira	M	NO	NO	NO	NO FOLLOW UP
24/07	10/8	46	F	15 Years	Yes	H/o previous ulcer trauma	NO	Lt G toe	II	165 mg%	Pseudo	M	NO	NO	YES	HEALED
64/07	11/8	70	F	10 Years	NO	Ulcer, HT	NO	Rt 3rd toe	II	116 mg%	Pseudo	M	NO	NO	YES	NO FOLLOW UP
70/08	12/8	61	M	20 Years	NO	PN Ulcer	NO	Lt dorsom	II	170 mg%	Kp	M	NO	YES	NO	NO FOLLOW UP
48/08	13/08	68	M	10 Years	Yes	Ulcer, PN	NO	Lt G toe	II	152 mg%	S.aureus,M.morganii	P	YES	NO	NO	NO FOLLOW UP
32/08	14/08	43	M	20	Yes	HT, PN, Ulcer trauma	Yes	Rt G-toe	II	272 mg%	Pseudo,E,coli	P	NO	YES	YES	NO FOLLOW UP
77/08	15/08	57	F	2	Yes	Ulcer, Amp, trauma	NO	Rt toe	I	100 mg%	Strep.pyogenes	P	NO	NO	NO	HEALED
71/08	16/08	47	M	2 Months	NO	trauma	NO	Rt G-toe	II	_	Enterococci	M	NO	NO	NO	NO FOLLOW UP
73/08	17/08	50	F	3	NO	Ht, Ulcer, Amp	NO	Rt plantor	III	192 mg%	S,aureus	M	NO	NO	NO	NO FOLLOW UP
88/07	18/08	58	M	2	NO	Uas, PN	NO	Rt ankle	II	_	K.oxytoca	M	NO	NO	NO	HEALED
	19/08	39	M	4 Years	Yes	Ulcer	NO	Rt G-toe	II	_	S.aureus ,Kp	P	NO	YES	NO	NO FOLLOW UP
73/07	20/08	57	M	12	Yes	Ht, PN, Ulcer, Amp	NO	Rt G-toe	III	290 mg%	Strep.pyogenes	P	NO	NO	NO	NO FOLLOW UP
22/08	21/08	64	F	7	Yes	PN Amp	NO	Lt G toe	II	197 mg%	K.oxytoca,Pseudo	P	NO	YES	YES	NO FOLLOW UP
72/06	22/08	55	M	8	NO	Ht, PN, Ulcer	NO	Lt ankle	II	148 mg%	S,aureus,P.mira	P	YES	NO	NO	FLAP SURGERY
8/7	23/08	40	M	6	Yes	PN, Amp trauma	NO	Gt G toe	II	_	Pseudo	M	NO	NO	YES	NO FOLLOW UP
95/08	24/08	50	F	4	Yes	HT, PN	NO	Rt foot	II	209 mg%	Enterococci	M	NO	NO	NO	HEALED
13/08	25/09	72	F	14 Years	Yes	HT, Retinopathy ulcer	NO	Lt G toe	II	217 mg%	CONS,E.coli	P	NO	YES	NO	FLAP SURGERY
95/08	26/09	84	M	10	Yes	PN Ulcer	NO	Lt G toe	II	159 mg%	E.coli	M	NO	NO	NO	NO FOLLOW UP
76/08	27/09	48	M	10	Yes	Ulcer, Amp	NO	Rt great toe,	II	202 mg%	S.aureus ,Kp	P	NO	YES	NO	HEALED
_	28/09	72	M	22 Years	Yes	HT, AMP	NO	Lt dorsom	II	319 mg%	S,aureus	M	YES	NO	NO	NO FOLLOW UP
8/8	29/09	61	F	11 Years	NO	PN	NO	Rt dorsom	II	_	C.freundi,CONS	P	NO	NO	NO	NO FOLLOW UP
29/08	30/09	54	M	7 Years	NO	HT Vas	NO	Lt G toe	II	98 mg%	Pseudo	M	NO	NO	YES	REG DRESSING
_	31/09	69	M	10	Yes	HT, Vas, PN, Ulcer, Amp	Yes	Rt dorsom	III	498 mg%	S,aureus,Pseudo	P	YES	NO	NO	AMPUTATION
27/08	32/09	62	M	5	NO	HT, PN	NO	Rt ankle	II	244 mg%	S,aureus	M	YES	NO	NO	NO FOLLOW UP
28/08	33/09	41	F	2	NO	PN	NO	Lt 3rd toe	I	164 mg%	Pseudo	M	NO	NO	NO	HEALED
_	34/09	60	F	10	Yes	PN	NO	Rt platar	II	_	S,aureus	M	YES	NO	NO	REG.DRESSING
7/7	35/09	52	M	12	Yes	PN	Yes	Rt dorsom	II	83%	S,aureus	M	NO	NO	NO	HEALED
29/07	36/09	54	F	5 Months	NO	PN	NO	Lt dorsom	II	294 mg%	S,aureus,E.coli	P	YES	NO	NO	FLAPSURGERY
6/9	37/09	50	F	25 Years	Yes	Ulcer, Amp	Yes	Lt 2nd toe stamp	III	119 mg%	CONS,P.mira,Kp	P	NO	YES	NO	amputation
9/9	38/09	55	F	5 Years	NO	PN	NO	Rt G-toe	I	190 mg%	S.aureus	M	NO	NO	NO	HEALED
4/9	39/09	54	M	15 Years	Yes	HT, PN, Ulcer	Yes	Rt Little toe	II	173 mg%	Strep.pyogenes	M	NO	NO	NO	NO FOLLOW UP
8/8	40/09	67	M	10	Yes	Vas ulcer	NO	Rt G-toe	II	214 mg%	CONS	M	NO	NO	NO	NO FOLLOW UP
35/09	41/09	30	F	2 Years	NO	PN	NO	Rt G-toe	I	117 mg%	CONS	M	NO	NO	NO	HEALED
36/09	42/09	60	F	11 Years	Yes	Amp	NO	Rt G-toe	II	155 mg%	P.mira,E.coli	P	NO	YES	NO	FLAP SURGERY
37/09	43/09	50	M	4 Years	Yes	HT, Vas	NO	Lt G toe	II	232 mg%	S.aureus	M	NO	NO	NO	NO FOLLOW UP
24/09	44/09	50	F	8 Years	Yes	HT, Retinopathy	Yes	Lt dorsom	III	299 mg%	S.aureus ,Kp	P	YES	YES	NO	REG.DRESSING
74/09	45/09	39	M	1 Year	Yes	_	NO	Rt 2nd toe	I	124 mg%	Strep.pyogenes	M	NO	NO	NO	HEALED
40/09	46/09	55	F	4 Years	NO	HT	NO	Lt plantar	I	200 mg%	P.vulgaris	M	NO	NO	NO	HEALED
31/09	47/09	45	F	2 Years	Yes	PN, Ulcer	Yes	Rt G-toe	II	292 mg%	Pseudo,E,coli	P	NO	NO	YES	NO FOLLOW UP
52/09	48/09	75	F	12 Years	Yes	HT, Retinopathy	NO	Lt ankle	II	166 mg%	S.aureus	M	NO	NO	NO	FLAP SURGERY
57/09	49/09	58	F	4 Years	NO	HT	NO	Rt dorsom	II	214 mg%	S.aureus	M	NO	NO	NO	NO FOLLOW UP
53/09	50/09	75	F	15 Years	Yes	Amp,PN	NO	Rt G-toe	III	_	CONS,Pseudo	P	NO	NO	NO	NO FOLLOW UP
57/09	51/09	58	F	7 Years	Yes	HT, PN	NO	Lt dorsom	II	114 mg%	E.coli,CONS	P	NO	YES	NO	NO FOLLOW UP
59/09	52/09	65	F	11 Years	Yes	Ulcer, Vas dis	Yes	Rt ankle	III	184 mg%	S.aureus ,Kp,CONS	P	YES	YES	NO	AMPUTATION
60/09	53/09	65	M	2 Years	NO	HT	NO	Rt G-toe	I	112 mg%	Strep.pyogenes	M	NO	NO	NO	HEALED

61/09	54/09	55	F	4 Months	Yes	PN trauma	NO	Rt 2nd toe	I	97 mg%	KP	M	NO	NO	NO	HEALED
51/09	55/09	45	M	2 Years	Yes	PN HT	NO	Rt G-toe	II	_	S.aureus ,Kp	P	NO	NO	NO	FLAP SURGERY
64/09	56/09	69	F	12 Years	NO	Vas ulcer	NO	Lt G toe	II	107 mg%	P.mira,E.coli	P	NO	YES	NO	FLAP SURGERY
65/09	57/09	40	F	6 Months	Yes	PN trauma	NO	Rt ankle	I	124 mg%	Strep.pyogenes	M	NO	NO	NO	HEALED
43/09	58/09	79	M	12 Years	Yes	HT, Vas, Amp	Yes	Rt G-toe	III	412 mg%	S.aureus	M	YES	NO	NO	REG DRESSING
67/09	59/09	39	M	1 Year	Yes	PN	NO	Rt ankle	I	98 mg%	Enterococci	M	NO	NO	NO	NO FOLLOW UP
68/09	60/09	55	F	2 Years	Yes	PN, Retinopathi	NO	Lt dorsom	II	142 mg%	Pseudo,Kp	P	NO	YES	NO	REG DRESSING
69/09	61/09	71	M	14 Years	Yes	HT, Amp	NO	Rt 3rd toe	II	248 mg%	Kp,CONS	P	NO	NO	NO	FLAP SURGERY
73/09	62/09	53	M	7 Years	NO	Ulcer	NO	Rt dorsom	II	166 mg%	S.aureus	M	YES	NO	NO	REG DRESSING
71/09	63/09	63	F	11 Years	Yes	Retinopathy	NO	Rt G-toe	II	112 mg%	KP	M	NO	NO	NO	FLAP SURGERY
70/09	64/09	60	F	1 Year	NO	PN	NO	Lt foot	I	88 mg%	Pseudo,E,coli	P	NO	NO	YES	REG DRESSING
72/09	65/09	34	F	4 Months	Yes	PN	NO	Rt G-toe	I	107 mg%	Enterococci	M	NO	NO	NO	REG DRESSING
74/09	66/09	55	M	3 Years	Yes	Ht, Ulcer	NO	Rt dorsom	II	_	KP	M	NO	NO	NO	REG DRESSING
75/09	67/09	64	M	14 Years	Yes	HT, PN, Amp,Vas	Yes	Rt 2nd toe	III	226 mg%	S.aureus	M	YES	NO	NO	AMPUTATION
76/09	68/09	46	M	7 Months	Yes	trauma	Yes	Rt foot	I	122 mg%	S.aureus ,Kp	P	NO	YES	NO	HEALED
79/09	69/09	47	M	2 Years	Yes	HT	NO	Lt foot	I	107 mg%	S.aureus	M	NO	NO	NO	NO FOLLOW UP
80/09	70/09	45	F	4 Months	Yes	PN, trauma	NO	Rt G-toe	II	154 mg%	P.mirabilis	M	NO	YES	NO	REG DRESSING
78/09	71/09	65	M	17 Years	Yes	HT, PN, Amp	NO	Rt 2nd toe	II	104 mg%	Strep.pyogenes	M	NO	NO	NO	NO FOLLOW UP
81/09	72/09	58	M	7 Months	NO	trauma	NO	Lt foot	I	_	P.mira,E.coli	P	NO	NO	NO	REG DRESSING
43/09	73/09	41	M	2 Months	Yes	PN, trauma	NO	Rt ankle	I	99 mg%	S.aureus	M	NO	NO	NO	REG DRESSING
35/09	74/09	68	M	10 Years	Yes	PN, Ulcer, Amp	NO	Rt foot	IV	199 mg%	S.aureus ,Kp	P	NO	YES	NO	NO FOLLOW UP
_	75/09	65	F	10 Years	Yes	Vas, ulcer	NO	Rt G-toe	II	273 mg%	Pseudo	M	NO	NO	YES	REG DRESSING
26/07	76/09	57	M	11 Years	Yes	Ht, PN, Ulcer, Amp	NO	Rt G-toe	II	227 mg%	S.aureus	M	NO	NO	NO	NO FOLLOW UP
32/09	77/09	60	F	4 Years	NO	HT,PN	NO	Lt foot	II	180 mg%	Pseudo,CONS	P	NO	NO	NO	REG DRESSING
36/09	78/09	60	F	5 Years	Yes	HT,Amp	Yes	Rt G-toe	II	110 mg%	KP	M	NO	NO	NO	REG DRESSING
45/09	79/09	50	F	7 Years	Yes	PN Ulcer	NO	Rt G-toe	III	_	S.aureus	M	NO	NO	NO	NO FOLLOW UP
43/09	80/09	77	M	16 years	Yes	HT, PN, Ulcer	NO	Lt foot	II	217 mg%	Pseudo,E,coli	P	NO	YES	YES	REG DRESSING
41/09	81/09	53	M	4 Years	Yes	HT	NO	Rt foot	II	117 mg%	KP	M	NO	NO	NO	REG DRESSING
38/09	82/09	76	F	11 Years	Yes	PN, Vas Amp	NO	Rt G-toe	II	194 mg%	S.aureus	M	YES	NO	NO	REG DRESSING
22/09	83/09	45	M	7 Months	Yes	HT	NO	Lt G toe	II	122 mg%	K.oxytoca	M	NO	NO	NO	NO FOLLOW UP
23/09	84/09	60	F	2 Years	Yes	PN, trauma	NO	Lt foot	I	_	P.mira,E.coli	P	NO	NO	NO	NO FOLLOW UP
37/09	85/09	69	M	1 Year	NO	Ht trauma	NO	GT foot	II	145 mg%	Strep.pyogenes	M	NO	NO	NO	NO FOLLOW UP
56/09	86/09	55	M	2 Years	Yes	PN, HT	NO	Rt G-toe	II	_	E.coli	M	NO	NO	NO	NO FOLLOW UP
51/09	87/09	56	M	7 Years	Yes	HT, Vas Ulcer	NO	Rt 2nd toe	II	247 mg%	S.aureus	M	NO	NO	NO	NO FOLLOW UP
51/09	88/09	39	M	4 Months	Yes	Ulcer	NO	Lt G toe	I	114 mg%	K.oxytoca	M	NO	NO	NO	REG DRESSING
27/08	89/09	47	M	7 Years	Yes	Ulcer	NO	Lt foot	II	_	S.aureus	M	NO	NO	NO	REG DRESSING
97/08	90/09	71	F	10 Years	Yes	Amp,ulcer	NO	Rt G-toe	III	202 mg%	P.mira,Kp	P	NO	NO	NO	AMPUTATION
70/09	91/09	64	M	11 Years	Yes	HT, PN Amp	NO	Rt G-toe	II	_	S.aureus	M	NO	NO	NO	NO FOLLOW UP
41/09	92/09	55	F	4 Years	NO	ht	NO	Rt dorsom	II	147 mg%	P.mirabilis	M	NO	NO	NO	NO FOLLOW UP
47/09	93/09	74	F	12 Years	Yes	PN, HT, Retinopathy	Yes	Rt G-toe	III	226 mg%	S.aureus ,.Koxytoca	P	YES	NO	NO	REG DRESSING
34/09	94/09	60	F	7 Years	Yes	HT, PN, trauma	NO	Lt G toe	II	134 mg%	P.mirabilis	M	NO	NO	NO	NO FOLLOW UP
17/09	95/09	64	M	11 Years	Yes	HT, PN Amp	NO	Rt 2nd toe	II	_	KP	M	NO	NO	NO	NO FOLLOW UP
79/08	96/09	66	M	12 Years	Yes	HT, PN Ulcer	NO	Rt G-toe	III	119 mg%	S.aureus,E.coli	P	NO	NO	NO	NO FOLLOW UP
49/08	97/09	44	F	4 Months	NO	Ulcer	NO	Rt foot	I	176 mg%	S.aureus ,P,mira	P	YES	NO	NO	REG DRESSING
66/09	98/09	75	F	8 Years	NO	Ht Retinopathy	NO	Rt G-toe	II	150 mg%	K.oxytoca	M	NO	YES	NO	REG DRESSING
74/08	99/09	66	M	7 Years	Yes	PN HT AMP	NO	Rt G-toe	III	249 mg%	CONS,P.mira	P	NO	NO	NO	NO FOLLOW UP
54/09	100/09	60	M	6 Years	Yes	PN, trauma	NO	Rt G-toe	II	172 mg%	S.aureus	M	YES	NO	NO	REG DRESSING

.

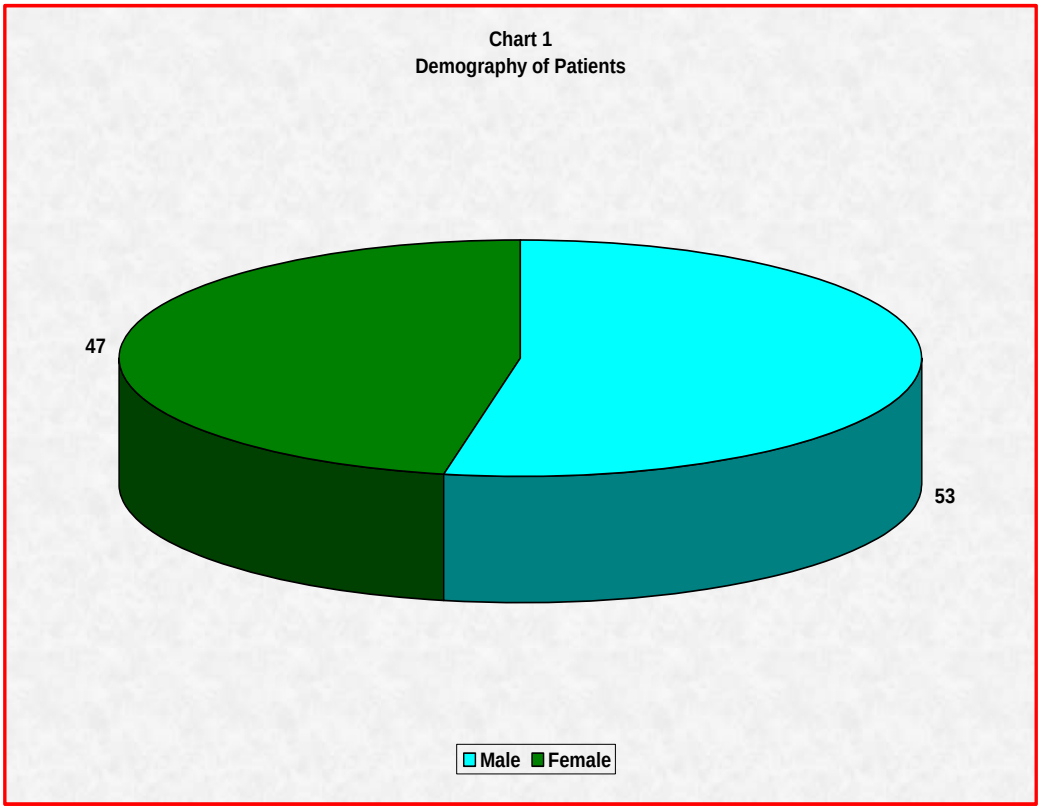


Chart 1
Demography of Patients
47
53
Male
Female

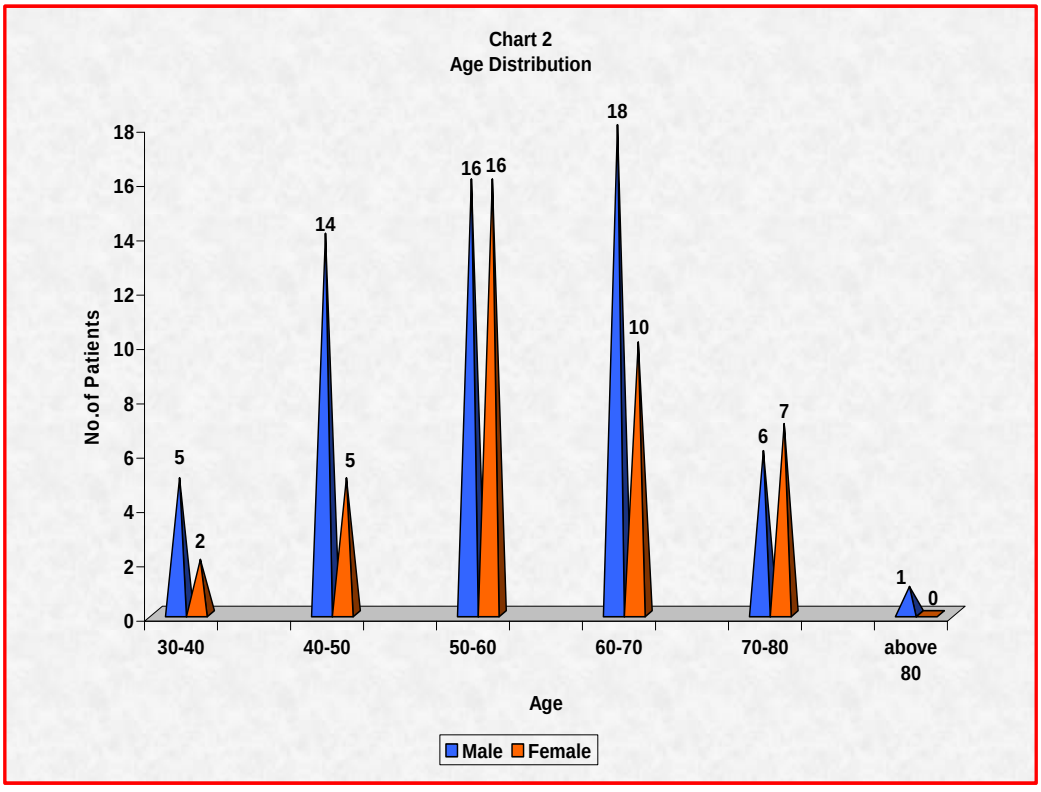


Chart 2
Age Distribution
No.of Patients
0
2
4
6
8
10
12
14
16
18
5
2
14
5
16
16
18
10
6
7
1
0
30-40
40-50
50-60
60-70
70-80
above 80
Age
Male
Female

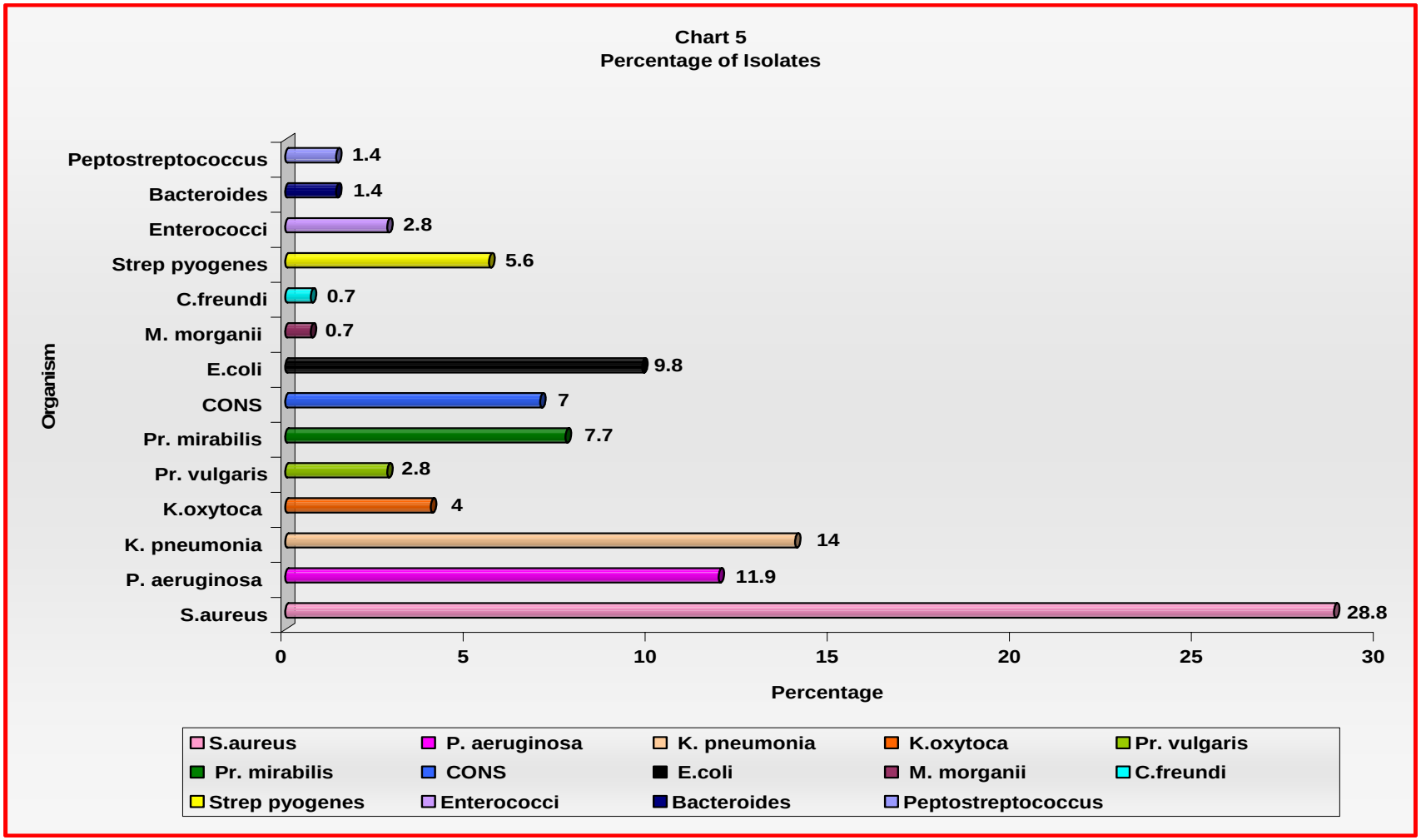


Chart 5
Percentage of Isolates
Organism
Peptostreptococcus
Bacteroides
Enterococci
Strep pyogenes
C.freundi
M. morganii
E.coli
CONS
Pr. mirabilis
Pr. vulgaris
K.oxytoca
K. pneumonia
P. aeruginosa
S.aureus
1.4
1.4
2.8
5.6
0.7
0.7
9.8
7
7.7
2.8
4
14
11.9
28.8
0
5
10
15
20
25
30
Percentage
S.aureus
P. aeruginosa
K. pneumonia
K.oxytoca
Pr. vulgaris
Pr. mirabilis
CONS
E.coli
M. morganii
C.freundi
Strep pyogenes
Enterococci
Bacteroides
Peptostreptococcus

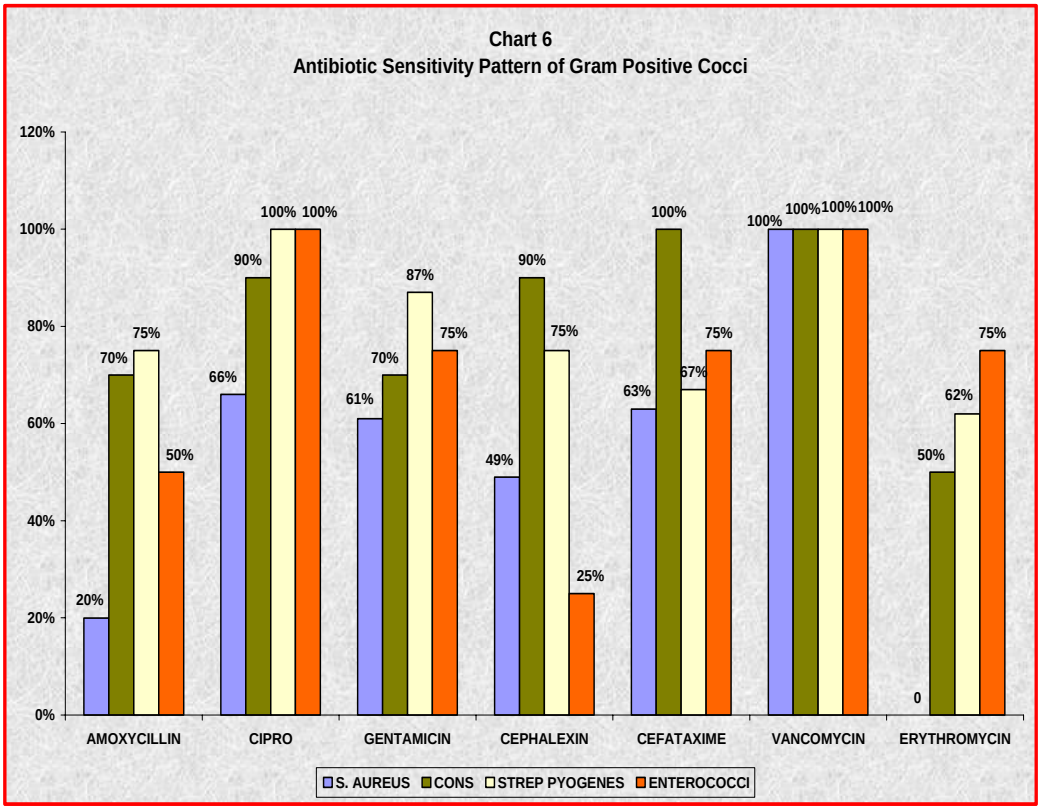


Chart 6
Antibiotic Sensitivity Pattern of Gram Positive Cocci
120%
100%
80%
60%
40%
20%
0%
20%
70%
75%
50%
66%
90%
100%
100%
61%
70%
87%
75%
49%
90%
75%
25%
63%
100%
67%
75%
100%
100%
100%
100%
0
50%
62%
75%
AMOXYCILLIN
CIPRO
GENTAMICIN
CEPHALEXIN
CEFATAXIME
VANCOMYCIN
ERYTHROMYCIN
S. AUREUS
CONS
STREP PYOGENES
ENTEROCOCCI

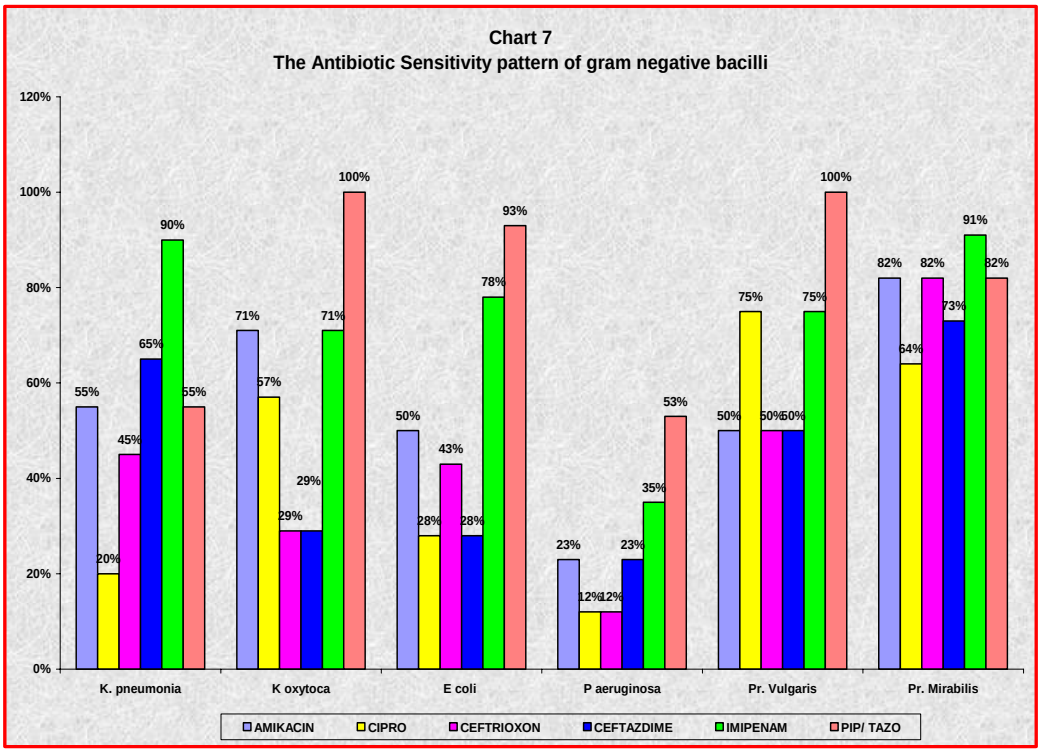


Chart 7
The Antibiotic Sensitivity pattern of gram negative bacilli
120%
100%
80%
60%
40%
20%
0%
55%
20%
45%
65%
90%
55%
71%
57%
29%
29%
71%
100%
50%
28%
43%
28%
78%
93%
23%
12%
12%
23%
35%
53%
50%
75%
50%
50%
75%
100%
82%
64%
82%
73%
91%
82%
K. pneumonia
K oxytoca
E coli
P aeruginosa
Pr. Vulgaris
Pr. Mirabilis
AMIKACIN
CIPRO
CEFTRIOXON
CEFTAZDIME
IMIPENAM
PIP/ TAZO

Chart 8
Percentage of MRSA Among S.aureus

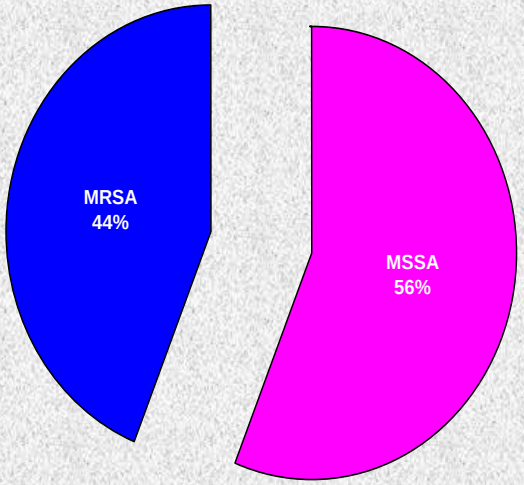


Chart 9
Percentage of ESBL and MBL among GNB